CHAPTER 6

THE SPOILAGE MICROBIOTA OF RAY (RAJA SP.) DURING ICE STORAGE UNDER DIFFERENT CONDITIONS: MOLECULAR IDENTIFICATION AND CHARACTERISATION OF THE SPOILAGE POTENTIAL

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Chapter 6. The spoilage microbiota of ray (*Raja* sp.) during ice storage under different conditions: molecular identification and characterisation of the spoilage potential.

Abstract

The dominant microbiota of ray stored on ice was systematically identified. Isolates grown on various media were identified by partial 16S rRNA, *gyr*B and *rpo*B gene sequencing. Microbiological shifts were observed during storage, ending in a dominance of especially members of the genera *Pseudomonas* and *Psychrobacter*. Most isolates could be identified by *rpo*B (*Pseudomonas* spp.) or *gyr*B gene sequencing as *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas psychrophila*, *Psychrobacter cibarius*, *Psychrobacter cryohalolentis*, *Psychrobacter glacincola* and *Psychrobacter immobilis*. Also species from the genera *Arthrobacter*, *Flavobacterium*, *Pseudoalteromonas*, *Shewanella* and *Staphylococcus* were detected during storage of ray. Subsequently, the spoilage potential of six selected isolates (*Flavobacterium tegetincola*, *Pseudomonas fluorescens*, *Pseudomonas psychrophila*, *Psychrobacter cibarius*, *Psychrobacter cryohalolentis* and *Shewanella frigidimarina*) was determined and quantified based on the presence of VOCs. Additionally, API ZYM and urease analyses determined the species' enzymatic capacity to contribute to spoilage by degrading lipids, amino acids and proteins and breaking down urea to ammonia. The six isolates were inoculated separately as pure cultures on gamma-sterilised ray. The inoculated samples were stored at 4°C and the production of VOCs by the pure strains on the ray matrix was identified via gas chromatography coupled to mass spectrometry (GC-MS). VOC production was quantified by selected ion flow tube mass spectrometry (SIFT-MS). The sensory profile of the selected species revealed that especially *Psychrobacter cibarius* and *Pseudomonas psychrophila* were able to produce higher concentrations of VOCs and might be responsible for the off-odours produced during spoilage of ray.

I. Introduction

In several European countries, ray (*Raja* sp.) encompasses the most commercialised elasmobranch fish species. In the North Sea, especially thornback ray (*R. clavata*), spotted ray (*R. montagui*) and blonde ray (*R. brachyura*) are caught in sandy coastal areas (Anonymous 2006). Since elasmobranchs (such as rays) contain concentrations of soluble components about twice as high as other seafood (Huss 1995), they are prone to rapid spoilage. Rays contain one to two percent urea in their muscles, blood, organs and skeleton, and have a

TMAO concentration two to three times higher than in cod (Elliot 1952; Huss 1995; Vyncke 1978). During spoilage, the stored urea will break down with the formation of ammonia. The main component causing rejection of elasmobranch fish and limiting the commercialization period is the fast increase in ammonia concentration (Finne 1992; Vyncke 1978). In elasmobranchs, the ammonia is formed mainly by enzymatic degradation of urea. The enzyme responsible for this activity is thought to be urease, present in certain bacteria (Vyncke 1978). However, a study by Mugica et al. (2007) found that the ammonia production was more correlated with the activity of the endogenous mechanisms involved in the degradation of proteins and NPN compounds, rather than with the activity of proteolytic microorganisms, meaning that the cause of ammonia production is still not known.

Although several studies have observed the effect of handling and processing procedures on the production of ammonia, nothing is known about the microbiota on elasmobranch fish and which microorganisms have the capacity to degrade urea to ammonia or produce other volatile organic compounds associated with spoilage.

The aim of this study was (1) to observe the shelf life of ray stored under different conditions, (2) to identify the dominant microbiota present on ray stored on ice to species level, and (3) to study the spoilage potential of these isolates by studying the volatile organic compound production of an inoculated pure culture on gamma sterile ray as detected by solid-phase micro-extraction gas chromatography coupled to mass spectrometry (SPME-GC-MS). The real-time quantification throughout the spoilage process of these chemical compounds was measured by selected ion flow tube mass spectrometry (SIFT-MS) analysis.

II. Materials and methods

2.1. Sampling of ray, lay out of the experiment and sampling during storage

Three blonde rays (*Raja brachyura*) were caught in august 2008 by beam trawling in the English channel. The catch was collected in large boxes. The rays were aseptically removed and were immediately put in a sterile bag and stored at 4°C until landed. The day after catch, the samples were transferred to the laboratory on ice. Three experiments were set up in the lab. The scheme of the set up and the microbiological analyses is given in Figure 6.1. Of two of the three rays the wings were carefully and aseptically removed (gutted) at day 1 (d1). One ray was further stored with the intestines (ungutted). The wings of the first ray (gutted) were stored on ice during a 9 day period with the skin. These wings were used to observe the

bacterial growth of skinned ray wings during storage on ice. At day 1 (d1) also the skin of one of the wings of the second gutted ray was removed aseptically with a sterile scalpel and forceps. The microbiological contamination degree and possible microbiological shifts during storage on ice between those two wings (with and without skin) were observed in order to study the effect of the skin on the shelf life of ray during storage. The third ray was kept on ice as a whole during 3 days. After those 3 days of storage (at d3), the wings were removed and one wing was left with skin. These wings were used to observe the differences between early gutted and late gutted rays on the shelf life and microbiota.

2.2. Cultivation of microorganisms

Several growth media were used to obtain a complete view of the ray-associated microbiota during storage on ice. The same media as in chapter 4 were used for the total aerobic psychotolerant count (APC): plate count agar (PCA, Oxoid), marine agar (MA, Difco) and modified Long and Hammer medium (LH) (Van Spreekens 1974). The enumeration of lactic acid bacteria (LAB) was performed on de Man Rogosa Sharpe medium (MRS, Oxoid) pH 6.5, and *Pseudomonas* species were enumerated on *Pseudomonas* Cetrimide Fucidine Cephaloridine (CFC, Oxoid) agar. *Enterobacteriaceae* were enumerated on violet red bile glucose (VRBGA, Oxoid) agar. Bacteria capable of producing hydrogen sulphide (black colonies) were enumerated on Lyngby iron agar (IA) (Atlas 2006).

For the microbiological analysis, 10 g of ray was transferred aseptically to a stomacher bag, 90 ml maximum recovery diluent (Oxoid) was added and the mixture was homogenized for two min. Samples (0.1 ml) of serial dilutions of the homogenates were spread on the growth media for enumeration. An incubation period of 1 day at 30°C (VRBGA), 5 days at 30°C (MRS), 3 days at 21°C (PCA, MA and CFC) or 5 days at 15°C (LH and IA) was used. Plating duplicates were made for every sample. After incubation, all typical colonies were counted. On IA, only the black colonies were counted as these represent the hydrogen sulphide producers.

2.3. DNA extraction

A selection of 284 isolates with different colony morphology were selected from PCA, MA, IA, LH and CFC media. These isolates were purified and DNA extraction was performed using the modified Flamm method as described in chapter 3. The DNA was stored at -20°C in HPLC water. The DNA concentration was measured upon usage by a Nanodrop 1000 spectrophotometer (Thermo Scientific).

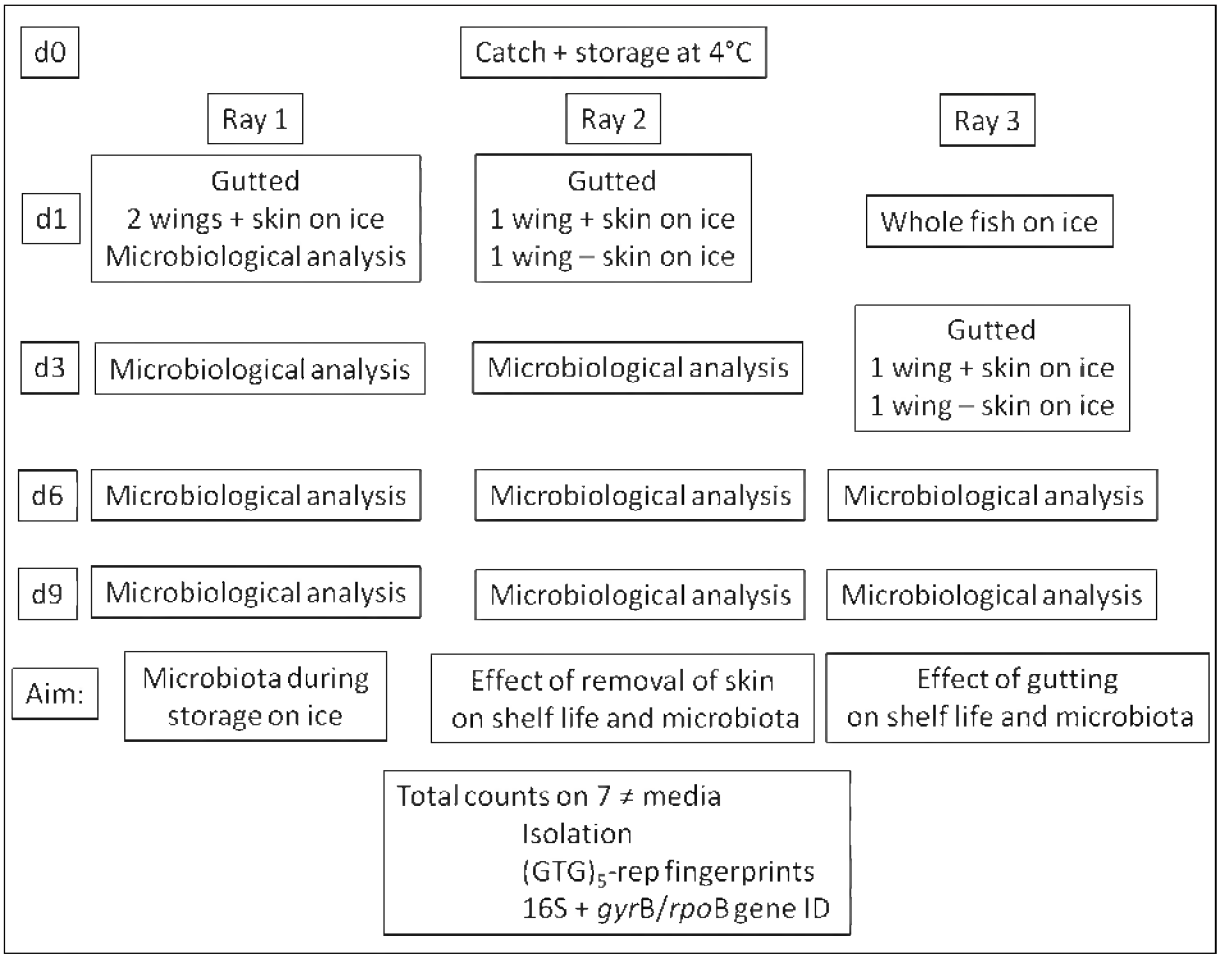

Fig 6.1. Scheme of the three experimental set ups with indication of the time intervals. T_0 = 1 day after catch = arrival at the laboratory, T_1 = after 3 days of aerobic ice storage, T_2 = after 6 days of aerobic ice storage, T_3 = after 9 days of aerobic ice storage.

2.4. Rep-PCR

The purified strains were clustered based on their $(GTG)_5$ rep-fingerprint. The PCR was performed as described in chapter 3. PCR products were size separated in a 1.5 % agarose gel in 1x TBE buffer (0.1M Tris, 0.1M Boric Acid, 2mM EDTA) at 120 V for 4 h. After ethidium bromide staining, the $(GTG)_5$ profiles were visualized under UV light and a digital image was captured using a G:BOX camera (Syngene). The resulting fingerprints were further analysed as described in chapter 3

2.5. Identification of the microbiological isolates by sequence analysis

Forty five representatives from the $(GTG)_5$ clusters were selected for identification. A 1500 bp fragment of the 16S rRNA gene (for all genera) and of the *gyr*B gene (for the genera *Shewanella*, *Psychrobacter* and *Pseudoalteromonas*) was amplified as described in chapter 3 and 4. For the species belonging to the genus *Pseudomonas*, a 1200 bp fragment of the *rpo*B gene was amplified as described in Tayeb et al. (2005). All PCR products were purified for

sequencing with a High Pure PCR product Purification kit (Roche) according to the manufacturer's instructions and stored at -20°C until sequencing. The quality and quantity of the purified PCR products was verified on 1.5% agarose gel.

Sequencing reactions, precipitation and sequencing on a ABI prism 3100 Genetic Analyzer (Applied Biosystems) was performed as described in chapter 3. The 16S partial sequences were mostly about 700 bp. The identification of phylogenetic neighbours was initially carried out by the BLAST (Altschul et al. 1997) and megaBLAST (Zhang et al. 2000) analysis of 16S partial sequences against the Eztaxon database of type strains with validly published prokaryotic names (Chun et al. 2007). The 50 16S sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was implemented at the *Eztaxon* server (http://www.*Eztaxon*.org/). The *gyr*B and *rpo*B sequences assembled with Vector NTI Advance 11 (Invitrogen corp.). A tentative identification was performed by a similarity search using the *Eztaxon* (16S; http://www.*Eztaxon*.org) and a FASTA (*gyr*B and *rpo*B) web search (http://www.ebi.ac.uk/tools/fasta33/nucleotide.html).

2.6. Characterisation of the spoilage potential: selection of the isolates

A selection of isolates was made based on their $(GTG)_5$-rep PCR fingerprints and partial 16S rRNA gene sequence identification. Representatives of large rep-clusters present when the fish was microbiologically spoiled were selected, as these were possibly most abundantly present on ray during storage and were possibly important for spoilage. In total 22 isolates were selected for API ZYM tests (Biomerieux): two *Arthrobacter* isolates, one *Flavobacterium* isolate, two *Pseudoalteromonas* isolates, nine *Pseudomonas* isolates, four *Psychrobacter* isolates, three *Shewanella* isolates and one *Staphylococcus* isolate. This test was used to determine their enzymatic activities for the following reasons: (1) to indicate the probable (biochemical) spoilage potential, and (2) to further clarify differences between the isolates in addition to $(GTG)_5$-rep fingerprints and sequence identification. In total, 19 enzymatic tests were performed: alkaline phosphatase; esterase (C4); esterase lipase (C8); lipase (C14); leucine arylamidase; valine arylamidase; cysteine arylamidase; trypsin; chymotrypsin; acid phosphatase; napthol-AS-Bi-phosphopydrase; α-galactosidase; β-galactosidase; β-glucoronidase; α-glucosidase; β-glucosidase; N-acetyl-β-glucosaminidase; α-mannosidase; and α-fucosidase. The isolates were cultured on plate count agar (Oxoid) or marine agar (Difco) at 21°C for 3 days, depending on the species. Further analysis and

interpretation was performed as described in chapter 5. These results were used to select the isolates for further study of the volatile compounds. Additionally, an urease test was performed on the same 22 isolates. This test was performed on Christensen's urea agar (Fluka) according to the manufacturers' guidelines with the exception of an incubation of 24 hours at 21°C. Isolates with the same $(GTG)_5$–rep PCR fingerprint, same sequence identification, and same API ZYM results were considered to be the same isolate (as was performed for shrimp in chapter 5).

2.6.1. Sample inoculation and storage

For each isolate, 300g of fresh ray was cut in 10g pieces, frozen en sent on dry ice for gamma sterilization in plastic stomacher bags. A minimal radiation dose of 25kGy was applied to completely sterilize the fish pieces. Afterwards, the fish was defrosted and aseptically transferred to sterile 2L bottles for air storage at 4°C. The bottles were inoculated resulting in concentration of 10^5 cfu/g of the selected pure strains. One bottle contained unirradiated ray pieces to compare the influence of radiation on the production of volatile organic compounds. Another bottle was filled with sterile ray which was not inoculated and was used as a control. All bottles were stored at 4°C for 5 days (T_4) until the end of the experiment. Samples were taken daily starting the day of inoculation (T_0) for bacterial enumeration and to identify (GC-MS) and quantify (SIFT-MS) the volatiles.

2.6.2. Total counts of inoculated samples and pH

The growth of the bacterial strains on the samples and pH of every sample was measured daily. For the microbiological analysis, 10 grams of ray were transferred aseptically to a stomacher bag, 90 ml of maximum recovery diluent (Oxoid) was added and the mixture was homogenised for 2 min. Samples (0.1 ml) of serial dilutions of the homogenates were spread on modified plate count agar or marine agar (depending on the species) for enumeration. An incubation period of 3 days at 21°C was used. Duplicates were made for every sample. After incubation all colonies were counted. The pH of every sample was performed by mincing 5 to 10 grams of ray sample and measuring the pH by using a pH meter (Mettler Toledo).

2.6.3. Identification of VOCs by GC-MS

Every other day starting at T_0, ray from each inoculated and control sample (sterilized and not sterilized) stored in air were aseptically prepared for SPME-GC-MS in order to identify all volatile compounds produced by the strains. The preparation of samples and conditions of the analysis were identical to those described in chapter 5 except that the SPME CTC PAL

autosampler (Agilent Technologies, Diegem, Belgium) equipped with a new 2 cm x 50/30 µm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) coated fibre (Supelco, Bellefonte, USA) was inserted through the PTFE septum for headspace (HS) extraction for 30 min at 50 ± 0.1 °C agitated at 500 rpm. Before use, the fibre was conditioned in a combiPAL conditioning station during 1h at 270°C as recommended by the manufacturer. After each desorption (10 min at 260°C in split less mode), the fibre was post-conditioned (20 minutes at 250°C) to avoid carry-over problems. In total, 33 compounds (Supplementary table 6.1.) were selected based on the SPME-GC-MS results (Table 6.4) and a preliminary research with SPME-GC-MS in combination with additional compounds generally found in fish spoilage (Duflos et al. 2006; Olafsdottir et al. 2005). Components present in all samples including the control (not inoculated samples) were not selected, they were considered natural sensory compounds of the matrix. Real-time quantification of these 33 compounds was performed using SIFT-MS.

2.6.4. Real-time quantification of the identified VOCs by selective ion flow tube mass spectrometry (SIFT-MS)

Every day during five days, starting with T_0, 50.0 ± 0.5g of ray from each inoculated and control sample, stored in air at 4°C, was aseptically taken for VOC quantification. The sample preparation, SIFT analysis and further interpretation was identically performed as in chapter 5. The supplementary table 6.1. shows the ionized masses used for quantification.

III. Results

3.1. Microbiological analysis of ray during storage on ice

Several media (general and specific) were used to obtain a complete view of the total microbiota on ray stored aerobically on ice. Figure 6.2 shows the microbiological counts on all media for ray 1 during storage on ice with skin. At d1, approximately 4 log was counted on PCA, while on MA and LH nearly 5 log was counted. On CFC, the number of *Pseudomonas* spp. was 3.2 log cfu/g. Lactic acid bacteria (MRS) were absent in 10g of ray during most of the study. *Enterobacteriaceae* (VRBGA) were absent in 10 g of ray during the whole study independent of the storage conditions. On iron agar, 2.5 log of H_2S producers were counted. During storage, microbiological counts increased and after 9 days of ice storage (d9) an increase of nearly 2 log with the counts at arrival at the laboratory (d1) was observed on all media.

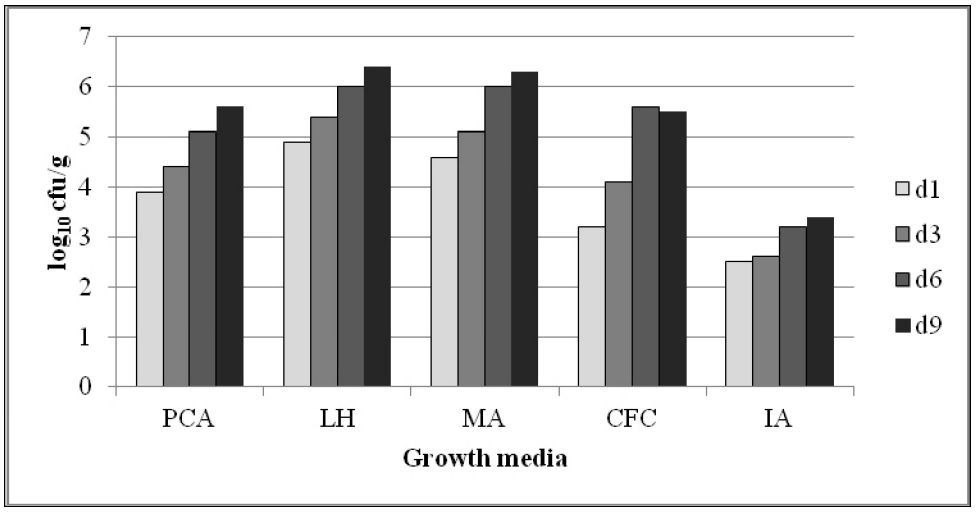

Fig 6.2. Total counts (log_{10} cfu/g) of gutted ray with skin during storage on ice on plate count agar (PCA), Long and Hammer medium (LH), marine agar (MA), *Pseudomonas* cetrimide fucidine cephaloridine (CFC) and Lyngby iron agar (IA). d1= arrival at the laboratory, 1 day after catch and storage on ice, d3= 3 days after catch and storage on ice, d6= 6 days after catch and storage on ice, d9 = 9 days after catch and storage on ice.

Effect of skinning and direct gutting on the shelf life

Table 6.1 shows the microbiological counts of ray 2 with and without skin. However, since analyses were performed on only a few samples, only possible trends can be demonstrated. Results show that deskinning lowers the total microbiological count on ray slightly. A small decrease in microbiological count observed after skinning. Eight days after skinning (at d9), the microbiological counts on the ray with skin seemed to increase and became slightly higher than on the skinned ray. This was also noticed for H_2S producers on IA. Late gutting showed a slightly higher total aerobic psychotolerant count nine days after catch (d9) compared with early gutting. Also the number of *Pseudomonas* spp. on CFC, the lactic acid bacteria (MRS) and sulphide producers (IA) showed a slightly higher total count with the early gutted ray with skin. When the skin was removed of the late gutted ray sample, total counts decreased a little and remained within a standard plate counting error of 0.5 log during further storage, while the total count on the not skinned ray increased between d6 and d9.

Table 6.1. Microbiological counts (log_{10} cfu/g) on various growth media of ray 2 and 3 stored on ice early and late gutted, with and without skin at d3 (3 days of ice storage), d6 (6 days of ice storage) and d9 (9 days of ice storage). PCA= plate count agar, LH= modified Long and Hammer medium (Van Spreekens, 1974), MA= marine agar, CFC= *Pseudomonas* Cetrimide Fucidine Cephaloridine, MRS= Man Rogosa Sharp medium, VRBGA= Violet Red Bile Glucose agar and IA= Iron agar (Atlas, 2006). - = not performed.

Media	Immediate gutted ray with skin (Ray 2)			Immediate gutted ray without skin (Ray 2)			Late gutted ray with skin (Ray 3)		Late gutted ray without skin (Ray 3)	
	d3	d6	d9	d3	d6	d9	d6	d9	d6	d9
PCA	4.7	5.1	5.1	4.3	4.3	5.9	5.3	6.4	5.3	5.7
LH	5.8	6.2	6.2	5.1	5.2	6.6	6.6	7.3	6.2	6.7
MA	5.4	5.9	6.0	5.0	5.1	6.6	6.5	7.3	6.1	6.5
CFC	4.8	5.0	5.0	3.7	4.2	5.9	5.5	6.4	5.0	5.5
MRS	<10	<10	<10	<10	<10	1.3	<10	2.1	1.5	1.8
VRBGA	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
IA	3.1	3.3	3.3	2.5	-	3.7	3.8	4.8	3.9	4.2

3.2. Molecular identification of the isolates of ray

In total 284 colonies with different colony morphology were isolated from PCA, MA, LH, CFC and IA. Each purified isolate was $(GTG)_5$-rep-fingerprinted. The pattern of all isolate showed great variety, with some large clusters of related isolates (similarity above 66%). Identification of those large clusters, with special attention to these isolates present at the end of storage, was important as those are part of the most abundantly recovered microbiota. From the $(GTG)_5$-rep-fingerprint fingerprints, 56 representatives were selected and identified based on their partial 16S rRNA gene sequence. Using partial 16S rRNA gene analysis, mainly species complexes could be found after BLAST search within the *Eztaxon* database. In total, 165 isolates present at d3 to d9, could be identified. Most of the identified isolates belonged to the genera *Pseudomonas* (39%), *Psychrobacter* (20%), *Pseudoalteromonas* (15%), *Flavobacterium* (10%) and *Shewanella* (10%). Other genera present during storage were *Arthrobacter* and *Staphylococcus* (Table 6.3). The remaining 119 of 284 isolates either had an unique $(GTG)_5$-rep-fingerprint or were not able to grow during ice storage and were therefore not identified. Further identification to species level (Table 6.3) was obtained via *gyr*B gene

Table 6.2. Summarizing table with all the identified species and genera found on ray during aerobic iced storage, with the percentage of similarity compared to the FASTA (*gyr*B and *rpo*B) websearch and the Eztaxon (16S) database. In the following columns give the number of identical isolates found, indicate the sample from which they were found (with/without skin, early/late gutted) and the time points during storage. d_1= present at arrival at the laboratory, 1 day after catch, d_3= present after 3 days of ice storage, d_6= present after 6 days of storage, d_9= present after 9 days of storage. With "x" the presence and abundance of the species is indicated on the sample.

Isolate name	Tentative phylogenetic neighbour (accession number)	Similarity (%)	# isolates	Time points	With/without skin		Gutted	
					With	Without	Early	Late
Arthrobacter								
2.42	*A. antarcticus* (AM931709)	98,7 (16S)	2	d_1, d_9	x	x	x	
1.154	*A. cryotolerans* (GQ406812)	98,1 (16S)	2	d_9			x	
2.200	*A. psychrochitiniphilus* (AJ810896)	98,8 (16S)	3	d_1, T_2	x		x	
Flavobacterium								
3.101	*F. frigoris* (AJ557887)	97,6-98,1 (16S)	3	d_1-d_9	x	xx	xx	x
3.92	*F. tegetincola* (U85887)	98,7-99,6 (16S)	13	d_1-d_9	xx	x	xx	x
Pseudoalteromonas								
3.4	*Psa. nigrifaciens* (FR668569)	99,5-97,5 (*gyr*B)	25	d_1-d_9	x	x	x	x
Pseudomonas								
1.9	*Ps. fluorescens* (AJ717451)	96,1 (*rpo*B)	4	d_1-d_9	xx	x	x	x
3.91	*Ps. fluorescens* (AJ717451)	99,6 (*rpo*B)	32	d_6-d_9	x	x	xx	x
1.172	*Ps. fragi* (AJ717444)	93,7-92,8 (*rpo*B)	12	d_1-d_9	x	x	x	x
2.102	*Ps. fragi* (AJ717444)	97,1 (*rpo*B)	4	d_1,d_9	x		x	
2.275	*Ps. mandelii*[1] (AJ717435)	95,0 (*rpo*B)	13	d_1-d_9	xx	x	x	
2.250	*Ps. vancouverensis*[9] (AJ717473)	96,4 (*rpo*B)						
1.155	*Ps. xanthomarina*[1] (FN554765)	99,5 (*rpo*B)						
1.135	*Ps. psychrophila*[1](AJ717464)	97,8 (*rpo*B)						
Psychrobacter								
3.85	*Psb. cibarius* (FR668579)	97,4-97,7 (*gyr*B)	8	d_6-d_9	x	x	x	x
2.256	*Psb. cryohalolentis* (DQ143922)	96,7 (*gyr*B)	3	d_6-d_9	x	x	x	
2.112	*Psb.glacincola* (DQ143926)	98,9 (*gyr*B)	6	d_6-d_9	x	x	x	
3.128	*Psb. immobilis* (DQ143927)	94,2-99,4 (*gyr*B)	16	d_6-d_9	xx	x	xx	x
Shewanella								
2.175	*S. frigidimarina*(AF014947)	98,4-98,5 (*gyr*B)	14	d_6-d_9	x	x	x	x
2.23	*S. putrefaciens* (AF005669)	98,7 (*gyr*B)	2	d_6	x			x
Staphylococcus								
2.35	*Staphylococcus* sp. (L37605)	95,2 (16S)	1	d_9		x	x	
3.222	*St. warneri* (L37603)	100 (16S)	2	d_9	x	x		x

[9] These isolates were all identified as *Pseudomonas psychrophila*, based on their partial 16S rRNA gene sequence and their $(GTG)_5$-rep fingerprint.

sequencing for the isolates belonging to the genera *Pseudoalteromonas*, *Psychrobacter* and *Shewanella* and via *rpo*B gene sequencing for the genus *Pseudomonas*.

3.3. Characterisation of the spoilage potential of the selected isolates

3.3.1. API ZYM and urease results

The API ZYM and urease results of the 22 isolates revealed differences in enzymatic activity. Table 6.3 shows the different enzymatic activities of the isolates. Differences between genera and between species were observed. The selected species of the genus *Arthrobacter* [*Arthrobacter antarcticus* (isolate 2.42) and *Arthrobacter cryotolerans* (isolate 1.154)] showed some enzymatic activity. They were able to degrade short (C4) to medium chain (C8) lipids and to hydrolyse leucine arylamidase. *Arthrobacter antarcticus* (isolate 2.42) was able to break down urea due to urease activity, an enzymatic activity which is very important in elasmobranch fish spoilage. The species representing the genus *Flavobacterium* (*Flavobacterium tegetincola*) appears to have a lot of enzymatic activity. The isolate shows phosphatase and lipase activity, and is able to hydrolyse not only amino acids but also proteins (α-chymotrypsin). *Pseudoalteromonas nigrifaciens* has positive phosphatase activity and is able to break down short to medium chain lipids. The species was able to hydrolyse leucine arylamidase and possesses urease activity.

The isolates of *Pseudomonas* only show positive hydrolysis of all tested amino acids (leucine arylamidase), and *Pseudomonas fluorescens* is able to hydrolyse valine arylamidase and to degrade urea to ammonia via urease activity. Both *Psychrobacter* isolates have positive phosphatase activity, have some lipolytical activity (esterase lipase) and are able to break down urea.

Shewanella frigidimarina shows phosphatase and lipolytic (esterase and esterase lipase) activity and hydrolysis of leucine arylamidase. The urease activity was variable between different isolates all identified as *Shewanella frigidimarina*. The *Staphylococcus* species in this study showed positive acid phosphatase activity and was able to degrade lipids (C4 and C8 chains). These phenotypical characteristics combined with the genotypic differences of the $(GTG)_5$- rep profiling resulted in the selection of 6 isolates, i.e., one *Flavobacterium* (isolate 3.92) , two *Pseudomonas* (isolates 1.9 and 1.135), two *Psychrobacter* (3.85 and 2.256) and one *Shewanella* (2.175) isolate(s).

Table 6.3. Molecular identification and enzymatic activities (via API ZYM and urease tests) of the selected isolates from ray during storage. 1: alkaline phosphatase, 2: acid phosphatase, 3: napthol-AS-Bi-phosphopydrase, 4: esterase (C4), 5: esterase lipase (C8), 6: lipase (C14), 7: leucine arylamidase, 8: valine arylamidase, 9: cysteine arylamidase, 10: trypsin, 11: α-chymotrypsin and 12: all carbohydrates grouped (α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase). V= variable, *= selected for further analysis.

ID (gene)	APIZYM results												Urease
	Phosphatases		Lipases				Hydrolysis of amino acids/proteins					Carbohydrates	
	1	2	3	4	5	6	7	8	9	10	11	12	
Arthrobacter													
Arthrobacter spp. (16S)	+	V	-	+	+	-	+	-	-	-	-	+[10]	V
Flavobacterium													
F. tegetincola (16S)*	+	+	+	+	+	+	+	+	+	-	+	+[11]	-
Pseudoalteromonas													
Psa. nigrifaciens (*gyr*B)	+	+	+	+	+	-	+	-	-	-	-	-	+
Pseudomonas													
Ps. fluorescens (*rpo*B)*	-	-	-	-	-	-	+	+	-	-	-	-	+
Ps. psychrophila (*rpo*B)*	-	-	-	-	-	-	+	-	-	-	-	-	-
Psychrobacter													
Psb. cibarius (*gyr*B)*	+	+	+	-	+	-	+	+	-	-	-	-	+
Psb. cryohalolentis (*gyr*B)*	+	+	+	-	+	-	+	-	-	-	-	-	+
Shewanella													
S. frigidimarina (*gyr*B)*	+	+	+	+	+	-	+	-	-	-	-	-	V
Staphylococcus													
Staphylococcus spp. (16S)	-	+	-	+	+	-	-	-	-	-	-	-	-

3.3.2. Total counts and pH

Figure 6.3 shows the results of bacterial growth of the inoculated sterile ray. The counts of the inoculated bacteria were 10^5 to 10^6 cfu/g after inoculation (at T_0). The unirradiated sample had a TVC of 5,62 log cfu/g, which is approximately the same as for the inoculated samples. After five days of storage at 4°C (T_4), nearly every inoculated sample except for *Flavobacterium tegetincola* (isolate 3.92) and *Psychrobacter cryohalolentis* (isolate 2.256) exceeded 10^7 cfu/g, also the TVC of the unirradiated control sample was nearly 10^8 cfu/g, while the TVC on the irradiated control sample was below the detection limit. The pH of the inoculated samples at T_0 was measured between 6.29 and 6.51. At T_4 the pH of the inoculated

[10] Positive for α-glucosidase

[11] Positive for N-acetyl-β-glucosaminidase

samples was between 6.79 and 7.18. These values are however much lower than the pH of the unirradiated control sample which reached a pH of 8.61 at T_4.

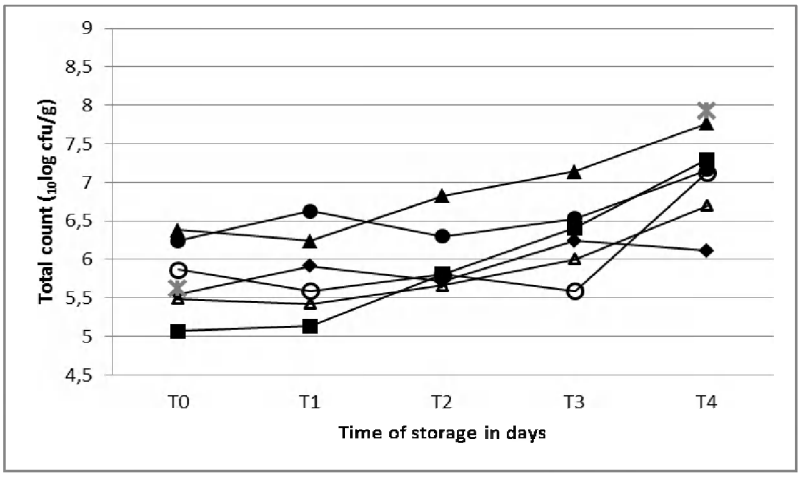

Fig 6.3. Growth of the bacterial inoculated isolates on chilled ray during storage at 4°C in air. The bacterial counts are given in $\log_{10}$ cfu/g. ◆= *Flavobacterium tegetincola* (isolate 3.92), ■= *Shewanella frigidimarina* (isolate 2.175), △= *Psychrobacter cryohalolentis* (isolate 2.256), ▲= *Psychrobacter cibarius* (isolate 3.85), ○= *Pseudomonas fluorescens* (isolate 1.9), ●= *Pseudomonas psychrophila* (isolate 1.135), ✳= Control unirradiated, T_0= day of inoculation, T_1= 1day of storage, T_2= 2 days of storage, T_3= 3 days of storage, T_4= 4 days of storage.

3.3.3. Volatile compounds

Table 6.4 shows the results of the GC-MS analysis of the inoculated pure strains on irradiated ray stored at 4°C. The compounds marked with an asterisk clearly increased in concentration during storage and were selected for further analysis with the SIFT-MS. Supplementary table 6.1 shows the 33 VOCs selected by GC/MS analysis and by literature search for further analysis during storage of the inoculated ray samples. Every day a quantitative SIFT-MS analysis for these 33 VOCs was performed on the inoculated samples and the control samples stored at 4°C. In the sample inoculated with *Shewanella frigidimarina* (isolate 2.175), no significant production of VOCs was detected after 5 days. Also the sample inoculated with *Flavobacterium* (isolate 3.92) showed limited VOC production, only 99 µg/m³ H_2S was produced, a concentration nearly four times above the olfactory threshold of 25.7µg/m³ as described by Devos et al. (1990). However, this sample reached the lowest TVC of all inoculated strains (6.2 log) which could be the cause of the low production. Several VOCs were detected and quantified in the samples inoculated with *Psychrobacter* and *Pseudomonas* isolates, however this production depended on the isolate. SIFT-MS results indicate that *Psychrobacter cryohalolentis* (isolate 2.256) was able to produce 1,3-butanediol, 2-hexanone, carbon disulphide, H_2S, 2-pentanamine, ammonia and also showed an increase in TMA production, independent of a low TVC (6.9 log). The highest number of compounds and at the

highest concentrations were produced by *Psychrobacter cibarius* (isolate 3.85) and *Pseudomonas psychrophila* (isolate 1.135). The most important compounds detected for *Psychrobacter cibarius* (isolate 2.256) were 1,3-butanediol, 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, acetoin, 2,3-butanedione, butanone, carbon disulphide, 2-pentanamine and acetic acid. For the inoculated samples with *Pseudomonas* isolates, *Pseudomonas fluorescens* (isolate 1.9) only produced 2-methylbutanal which is limited compared to *Pseudomonas psychrophila* (isolate 1.135) which has a similar TVC and produces 1,3-butanediol, 2-methylbutanal, carbon disulphide, H_2S, 2-pentanamine, DMA, ammonia and a low concentration of TMA. Aldehydes such as 2-methylbutanal (threshold: 123 µg/m³), 3-methyl-butanal (threshold: 8.12 µg/m³) and 2-methylpropanal (threshold: 123 µg/m³) were produced above the olfactory threshold in the inoculated samples. 2-methylbutanal was produced by *Pseudomonas psychrophila* (isolate 1.135) at the highest concentration (1033.1 µg/m³), followed by *Pseudomonas fluorescens* (502 µg/m³) and *Psychrobacter cibarius* (446.3 µg/m³). 2-methylpropanal (332.8 µg/m³) and 3-methylpropanal (700.1 µg/m³) were only produced by *Psychrobacter cibarius* (isolate 2.256) above the olfactory threshold of 123 µg/m³ and 8.12 µg/m³ respectively. The ketone 2,3-butanedione was also produced by *Psychrobacter cibarius* in a concentration of 586.5 µg/m³, much higher than the threshold (15.8 µg/m³). The sulphur compounds carbon disulphide and H_2S have a olfactory threshold of 302 µg/m³ and 25.7 µg/m³ respectively. Carbon disulphide was produced by both *Psychrobacter* species above this threshold (*Psychrobacter cibarius* 893 µg/m³ and *Psychrobacter cryohalolentis* (302.4µg/m³). The concentration of TMA increased at T_4 for *Pseudomonas psychrophila* (205.8 µg/m³) and *Psychrobacter cryohalolentis* (394 µg/m³), to concentrations clearly above the olfactory threshold of 5.88 µg/m³, indicating a possible production. However, this concentration is extremely low compared to the real TMA producers such as *Photobacterium* and is also much lower than the TMA concentration of the unirradiated ray (control) sample (202155µg/m³). Ammonia, a typical compound indicative of spoilage of ray, was produced by *Pseudomonas psychrophila* (4346.8 µg/m³) and *Psychrobacter cryohalolentis* (5139.8 µg/m³), both slightly above the threshold of 4073.8 µg/m³. However, it was not present on the unirradiated control sample. On the unirradiated ray sample also ethanol (1154.8 µg/m³), acetone (281.6 µg/m³), 2-nonanone (1200.3 µg/m³), 2-undecanone (66.9 µg/m³) and methyl mercaptan (912.2 µg/m³) was found, which were not detected on the inoculated samples. Several compounds produced by the inoculated samples were also produced on the not sterilized ray (control) sample but in much higher

concentrations: 2-methylbutanal (1155.3 µg/m³), carbon disulphide (381168.8 µg/m³), and all three amines (DMA: 68463 µg/m³, 2-pentanamine: 36592.6 µg/m³ and TMA 202155 µg/m³). Other compounds were not produced in the unirradiated control sample, namely 3-methylbutanal, 2-methylpropanal, acetic acid and surprisingly also ammonia.

IV. Discussion

Elasmobranch fish such as ray are known for their quick spoilage shortly after catch. This spoilage is possibly caused due to the high NPN fraction and subsequently the production of ammonia from the high urea content present in the fish. In this study, the dominant microbiota of ray stored on ice under different conditions is identified and their spoilage potential is unravelled. The microbiological analysis shows that one log difference was observed between PCA and marine media as previously noticed on various fish species including ray (chapter 3). This is due to the presence of halotolerant species unable to grow on PCA. At T_0, all samples were well within the microbiological limits of 10^5-10^6 cfu/g for fresh and precooked fish (Anonymous 1986). The microbiological analysis shows that the TVC on ray doesn't exceed 10^7 cfu/g after 9 days of storage except for late gutted ray stored with skin. However, all the ray samples were no longer considered suitable for consumption due to a strong ammonia smell. The absence of *Enterobacteriaceae* indicates that the ray samples were processed under hygienic conditions. Lactic acid bacteria (LAB), were only present after a few days of storage when the ray sample was stored unskinned and when the ray was not immediately gutted.

Skinning of the fish gave a lower total count on all media at three to six days after skinning compared to fish that had not been skinned. However, during further storage, total counts increased much faster resulting in a much higher total count. One of the functions of the skin of fish is protection against microbiological penetration, which can explain the steep increase in microbiological count after prolonged storage without skin. On the other hand, removing the skin decreases the total count at a short term, this because the slime layer contains a high amount of nitrogenous compounds, which provides nutrients for the microbiota on the skin and is therefore a microbiological contamination source. Therefore it might be useful to leave the skin on until purchase, however, more data should be obtained to make a correct statement.

Table 6.4. GC-MS results on irradiated ray inoculated with *Flavobacterium*, *Pseudomonas*, *Psychrobacter* and *Shewanella* strains and stored in air at 4°C. Analyses were performed after 5 days of storage at 4°C. The compounds marked with an asterisk showed an increase during storage and were incorporated in the SIFT-MS method for quantification.

Compound	*Flavobacterium tegetincola*	*Pseudomonas psychrophila*	*Psychrobacter cibarius*	*Psychrobacter cryohalolentis*	*Shewanella frigidimarina*
1,3-butanediol*				x	
1-hexen-3-ol		x			
1-penten-3-ol	x		x	x	x
1-undecene		x			
2,2,4,6,6 penta-methylheptane			x	x	
2,4-dimethyl-1-heptene	x	x	x	x	
2-butanone*			x		
2-ethyl-1-hexanol	x	x	x	x	x
2-ethyl-hexanal		x			
2-heptanone		x			x
2-hexanone*	x		x		x
2-methyl-1-pentene	x	x			
2-methylbutanal*	x				
2-nonanone*		x	x		x
2-pentanamine*		x			
2-pentanone*		x			
2-undecanone		x			
3,4-heptadiene	x				
3-hydroxy-2-butanone	x		x	x	x
3-methyl-1-butanol*		x	x	x	x
3-methylbutanal*	x		x	x	
4-methyl-heptane	x	x	x	x	
Acetone*	x	x	x	x	
Benzene			x	x	
Dimethyldisulphide*					x
Dimethyl sulphide*	x	x	x	x	xx
Ethanol*	x				
Ethylbenzene					
Eucalyptol	x	x	x	x	x
Methanethiol					xx
Methylisobutyl-ketone				x	
o-xylene		x			
Pentane*	x				
Styrene				x	
Toluene	x		x	x	x
Trimethylamine*	x	x		xx	xx

The results from early and late gutting are based on little data. Therefore, only assumptions can be made. It was noticed that especially the number of LAB and the amount of H_2S producers increased, which may contribute to an unacceptable sulphur odour. However, further study should necessary to verify this assumption.

Identification of the isolates (Table 6.3) shows that species from the genus *Pseudomonas*, *Psychrobacter*, *Pseudoalteromonas* but also *Flavobacterium* and *Shewanella* dominate the microbiota of fresh ray. During storage, microbiological shifts are noticed. At the beginning of storage (d1-d3), the fresh fish is mainly dominated by *Pseudomonas*, *Arthrobacter*, *Pseudoalteromonas* and *Flavobacterium* species. During further storage on ice (d6-d9), the number of isolates identified as *Arthrobacter* and *Flavobacterium* decrease, while the numbers of *Psychrobacter*, *Shewanella* and also a small number of *Staphylococcus* species increase. Species identified as *Arthrobacter* are present at the beginning of storage but are virtually competed out by *Pseudomonas* spp. and *Psychrobacter* species during storage. Also the storage conditions, have an influence on the microbiota present; *Shewanella putrefaciens* and *Staphylococcus warneri* were detected when the ray was gutted late, indicating a contamination from the intestines. Since *S. putrefaciens* is a strong SSO (Gram and Huss 1996; Molin and Stenström 1984), early and hygienic gutting of the fish is recommended for shelf life extension. Plating techniques (due to the species' obvious colony morphology (large, slimy, light brownish convex colonies) on Long and Hammer medium) showed that also *Pseudoalteromonas nigrifaciens* is present in higher numbers when the fish is stored ungutted. Not only *Pseudoalteromonas* but also *Psychrobacter* species are often associated with the gut microbiota of seafood (Fjellheim et al. 2007; Meziti et al. 2010; Oxley et al. 2002).

In this study, all the microorganisms identified during storage were marine food-related microorganisms. Most of them have been isolated before from food or seafood. Some of the genera are known seafood spoilage microorganisms, such as *Pseudomonas* sp. *Pseudomonas* has often been observed to dominate the microbiota of seafood stored aerobically under chilled conditions (Gennari et al. 1999; Koutsoumanis and Nychas 1999; Shewan et al. 1960; Stenström and Molin 1990; Tryfinopoulou et al. 2002), which leads them to be used as a spoilage indicator (Olafsdottir et al. 2006). However *Pseudomonas* species are considered to grow very rapidly and outgrow other genera (Moore et al. 2006). Species are often found to co-exist with other *Pseudomonas* sp. or other psychrothrops such as *Shewanella putrefaciens*

or *Psychrobacter immobilis* (Blackburn 2006). Two of the four *Pseudomonas* species mostly associated to food spoilage were also found in this study, namely *Pseudomonas fluorescens* and *Pseudomonas fragi*. Next to seafood spoilage, those species are also associated with spoilage of meat, poultry, milk and fresh produce (only *Pseudomonas fluorescens*). Spoilage by these proteolytic and lipolytic microorganisms is indicated by a slimy and musty appearance, the production of off-odours and at the end partial or complete degradation of the animal tissue (Blackburn 2006). In this study, the isolates identified via *rpo*B sequencing respectively as *Pseudomonas fluorescens* (isolate 1.9) and *Pseudomonas psychrophila* (isolate 2.256), did not show such a high odour production potential. This is in contrast to previous studies where the species was associated with the production of alcohols (methanol and ethanol), TMA, ammonia, ethyl acetate, ketones such as acetone and 2-pentanone or sulphur compounds (Chinivasagam et al. 1998; Edwards et al. 1987; Freeman et al. 1976; Nychas et al. 2007; Pittard et al. 1982; Reynisson et al. 2009; Schmitt and Schmidtlorenz 1992). In those previous studies, especially *Pseudomonas fluorescens* (isolate 1.9) was thought to have a high odour production potential, which has not been shown in this study. This lower VOC potential can be due to the low total counts of the isolates on the sterilized ray at the end of the SMPE-GC-MS and SIFT-MS analysis. The total count did not exceed 10^8 cfu/g, while a total count of 10^8-10^9 cfu/g is generally thought to be needed in order to start the excessive production of volatiles (Gram et al. 2002). Also the inoculation of one pure strain can make a difference. Another possibility is a wrong identification in the previous studies due to phenotypic identification, since this genus has suffered from severe identification problems in the past, which is still reflected in the current taxonomy (Tryfinopoulou et al. 2002). Discrepancy between the *rpo*B gene and 16S rRNA gene analyses gave different results for the same isolate (isolate 3.91) in this study, namely *Pseudomonas fluorescens* (99.6% similarity – *rpo*B) against *Pseudomonas gessardi* (97.7% similarity – 16S). Wrong identification of isolates associated to seafood spoilage might also be the reason why *Pseudomonas psychrophila*, identified by *rpo*B gene analysis, has not been associated with seafood spoilage despite his potential to produce VOCs such as alcohols, aldehydes, sulphur compounds, amines (including TMA) and ammonia as described in this study. *Pseudomonas psychrophila* is closest related to *Pseudomonas fragi*, a well-known spoiler. DNA-DNA hybridisation however showed that homology was too low to be the same species, resulting in a new species (Yumoto et al. 2001). The other *Pseudomonas* isolates found in this study identified by *rpo*B analysis as *Pseudomonas mandelii*, *Pseudomonas vancouverensis*, *Pseudomonas*

xanthomarina and *Pseudomonas psychrophila* (Table 3) were all clustered together based on their $(GTG)_5$-rep fingerprints. Also 16S rRNA gene analysis identified them all as *Pseudomonas psychrophila* with similarities between 97.4 and 100%.

The two test strains of *Pseudomonas* didn't show much enzymatic activities in the API ZYM test, only hydrolysis of amino acids. *Pseudomonas fluorescens* (isolate 1.9) was able to degrade urea.

Also the other genera (*Psychrobacter*, *Pseudoalteromonas*, *Flavobacterium* and *Shewanella*) found in this study have been associated to seafood and spoilage (see previous chapters and Bjorkevoll et al. 2003; Castell and Mapplebeck 1952; Chai et al. 1968; Jaffrés et al. 2009; Mejlholm et al. 2005; Paarup et al. 2002; Prapaiwong et al. 2009; Tsironi et al. 2009). Four different *Psychrobacter* species were found in this study, only one of which, *Psychrobacter immobilis*, is known to have a minor spoilage potential, producing a musty odour (Björkevoll et al. 2003; Mejlholm et al., 2005; Prapaiwong et al. 2009) and ammonia (Ozogul and Ozogul 2007). The other *Psychrobacter* species have not been associated to spoilage so far. The genus' importance in spoilage was also considered rather low since they seemed to be unable to compete with common spoilage microorganisms (Rodriguez-Calleja et al. 2005), which is in contrast with the results found in this study. This study indicates a co-dominance with *Pseudomonas* spp. on ray during aerobic ice storage and indicates that *Psychrobacter cryohalolentis* and especially *Psychrobacter cibarius* was able to produce VOCs (e.g. alcohols, acetoin and sulphur compounds) possibly associated to spoilage. This is in contrast to the spoilage potential of *Psychrobacter cibarius* on sterilized shrimp, as it does not produce any VOCs and therefore does not contribute to sensory spoilage (Chapter 5). *Psychrobacter* species were also positive for a large number of enzymatic activities such as phosphatases, medium chain lipid break down and hydrolysis of amino acids. Both species studied (*Psychrobacter cibarius* and *Psychrobacter cryohalolentis*) were also able to break down urea, an important characteristic concerning spoilage of elasmobranch fish.

Pseudoalteromonas nigrifaciens, formerly not associated with spoilage, seemed to be an effective spoiler of brown shrimp without preservatives producing several VOCs associated with spoilage as observed in chapter 5. However, the spoilage potential on ray was not further investigated. It is possible that the species is able to contribute to spoilage though.

Flavobacterium species, although present until the end of storage, were mainly found at the beginning of aerobic iced storage of ray and consisted of *Flavobacterium frigoris* and *Flavobacterium tegetincola*. Previous literature (Castell and Mapplebeck 1952) has observed

that some *Pseudomonas* species are able to inhibit *Flavobacterium*, a phenomenon which may happen on ray during iced aerobic storage as well. The same study indicated that *Flavobacterium* grew more slowly and produced less offensive odours than for instance *Pseudomonas*, which is confirmed in this study. They showed that some species were able to produce offensive odours from stale and sweet to putrid or faecal when inoculated as pure cultures on the fish matrix. Also a few were able to reduce trimethylamine oxide to TMA. Freeman et al. (1976) associated ethanol and dimethyldisulfide production with *Flavobacterium* species found during spoilage. Via SIFT-MS analysis in this study it was found that *Flavobacterium tegetincola* (isolate 3.92) only produces hydrogen sulphide during storage. The API test however did show that *Flavobacterium tegetincola* (isolate 3.92) has a large potential or enzymatic activity, whereas it was the only isolate able to hydrolyse proteins as well and was able to break down carbohydrates next to phosphatase, lipase and hydrolysis of amino acid activity. The isolate was however urease negative.

During aerobic storage on ice, a number of *Shewanella* species are able to outgrow on ray. Especially when the fish is stored ungutted, the typical SSO *Shewanella putrefaciens* will be present. Another species, identified as *Shewanella frigidimarina* (isolate 2.175) by *gyr*B gene sequencing did not significantly contribute to spoilage. However the species may contribute to spoilage due to enzymatic activities (phosphatase and lipase activity and hydrolysis of leucine arylamidase). Identification of this species by 16S rRNA gene sequencing resulted in a species complex with high similarity between the species *Shewanella vesiculosa*, *Shewanella frigidimarina* and *Shewanella livingstonensis*. Accordingly, *gyr*B analysis tentatively identified the isolates as *Shewanella frigidimarina* (similarity 98.4 – 98.5%). A recent described new species, *Shewanella arctica*, did suffer from the same species complex based on the 16S rRNA sequence (Kim et al. 2011). A comparison between our isolate's 16S rRNA gene sequence and the sequence of *Shewanella arctica* gave a similarity of 99.9%. Since there are not yet any *gyr*B sequences present in a database, we are unable to compare sequences, but it is very likely that the isolates found in this study on ray are *Shewanella arctica* rather than *Shewanella frigidimarina*.

The large differences between the VOC production on the inoculated samples and the not-sterilized control sample, can be explained as the VOC production of pure isolates on a sterilized matrix can be different than the production in co-existence with other microorganisms. This can result in benefits from microbiological interaction such as antagonism or metabiosis (Gram and Melchiorsen 1996; Gram et al. 2002). Also other

microorganisms which were not found or for which no VOC profile was determined, could contribute to spoilage and by doing so create a different VOC profile in natural contaminated ray. VOC analysis of co-inoculated strains as well as comparison with natural contaminated ray should be interesting for further research.

Gamma sterilization did not have an effect on the matrix itself as both control samples (sterilized and not sterilized) had the same SIFT-MS profile at T_0.

In conclusion, this study has contributed to the knowledge concerning the dominant microbiota on aerobic ice stored ray under different conditions. The spoilage potential of *Pseudomonas*, *Psychrobacter*, *Flavobacterium* and *Shewanella* isolates/species inoculated as pure cultures on sterile ray was studied. The sensory profile results indicate that especially *Pseudomonas* and *Psychrobacter* species may contribute to the off-odours produced during storage. However, due to the low counts of the inoculated isolates (below 10^8 cfu/g) at the end of the SIFT-MS analysis, all results are only indicative. The selected isolates may have a much higher spoilage potential than mentioned in this study, not only by VOC production but also by enzymatic activities. Both *Psychrobacter* species (*Psychrobacter cibarius* and *Psychrobacter cryohalolentis*) and *Pseudomonas fluorescens* were able to degrade urea to ammonia based on an enzymatic test. However SIFT-MS analysis showed that only *Psychrobacter cryohalolentis* and *Pseudomonas psychrophila* produced ammonia in concentrations above the olfactory threshold so that they could be observed by the human nose. This study might also indicate that the ammonia production possibly is caused by bacterial activity rather than autolytic processes, since no ammonia production was observed on the irradiated ray samples, in contrary of the production by some *Psychrobacter* and *Pseudomonas* isolates.

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Supplementary table 6.1. Mass-to charge Ratio, m/z, values of the characteristic product ions of the volatile compounds shown analysed by SIFT-MS using H_3O^+ , NO^+ and O_2^+ precursor ions.

Volatile compound	Precursor	m/z	Branching ratio (%)	K	Characteristic product ion
Alcohols					
1,2-butanediol	NO^+	89	100	3.90E -09	$C_5H_{11}O^+$
1,3-butanediol	NO^+	89	100	1.1E -09	$C_4H_9O_2^+$
ethanol	H_3O^+	47	100	2.70E -09	$C_2H_7O^+$
	H_3O^+	65		2.70E -09	$C_2H_7O^+.H_2O$
isobutyl alcohol	H_3O^+	57	100	2.70E -09	$C_4H_9^+$
	NO^+	73	95	2.40E -09	$C_4H_9O^+$
3-methyl-1-butanol	H_3O^+	71	100	2.8E -09	$C_5H_{11}^+$
	NO^+	69	10	2.3E -09	$C_5H_9^+$
2-propanol	H_3O^+	43	80	2.70E -09	$C_3H_7^+$
Aldehydes					
hexanal	NO^+	99	100	2.5E -09	$C_6H_{11}O^+$
	O_2^+	56	50	2.0E -09	$C_4H_8^+$
2-methylbutanal	H_3O^+	45	2	3.7E -09	$C_2H_5O^+$
	NO^+	57	2	3.2E -09	$C_4H_9^+$
3-methylbutanal	NO^+	85	100	3.0E -09	$C_5H_9O^+$
2-methylpropanal	NO^+	71	100	3.1E -09	$C_4H_7O^+$
Ketones					
acetoin	H_3O^+	89	100	3.0E -09	$C_4H_8O_2.H^+$
	H_3O^+	107		3.0E -09	$C_4H_8O_2.H^+.H_2O$
	NO^+	118	100	2.5E -09	$C_4H_8O_2.H^+.NO^+$
	O_2^+	88	20	2.5E -09	$C_4H_8O_2^+$
acetone	H_3O^+	59	100	3.90E -09	$C_3H_7O^+$
2,3-butanedione	NO^+	43	80	1.3E -09	$C_2H_3O^+$
butanone	NO^+	102	100	2.80E -09	$NO^+.C_4H_8O$
2-hexanone	NO^+	130	100	3.6E -09	$NO^+.C_6H_{12}O$
	O_2^+	100	5	3.4E -09	$C_6H_{12}O^+$
2-nonanone	H_3O^+	143	100	4.2E -09	$C_9H_{18}OH^+$
	H_3O^+	161		4.2E -09	$C_9H_{18}OH^+.H_2O$
	NO^+	142	2	2.7E -09	$C_9H_{18}O^+$
	NO^+	172	98	2.7E -09	$C_9H_{18}O^+.NO^+$
	O_2^+	142	30	3.2E -09	$C_9H_{18}O^+$
2-pentanone	NO^+	116	100	3.10E -09	$NO^+.C_5H_{11}O$

	NO^+	88	100	1.20E -09	$NO^+.C_3H_6O$
2-undecanone	H_3O^+	171	100	4.3E -09	$C_{11}H_{22}OH^+$
	H_3O^+	189		4.3E -09	$C_{11}H_{22}OH^+.H_2O$
	NO^+	200	100	3.4E -09	$C_{11}H_{22}O^+.NO^+$
	O_2^+	110	10	3.4E -09	$C_8H_{14}^+$
	O_2^+	127	5	3.4E -09	$C_9H1_9^+$
	O_2^+	152	5	3.4E -09	$C_{11}H_{20}^+$
	O_2^+	170	15	3.4E -09	$C_{11}H_{22}O^+$
Sulphur compounds					
carbon disulphide	O_2^+	76	100	7.0E -09	CS_2^+
dimethyl disulphide	H_3O^+	95	100	2.60E -09	$(CH_3)_2S_2.H^+$
	NO^+	94	100	2.40E -09	$(CH_3)_2S_2^+$
	O_2^+	61	10	2.30E -09	$CH_3CH_2S^+$
	O_2^+	94	80	2.30E -09	$(CH_3)_2S_2^+$
dimethyl sulphide	H_3O^+	63	100	2.5E -09	$(CH_3)_2S.H^+$
	NO^+	62	100	2.2E -09	$(CH_3)_2S^+$
	O_2^+	47	25	2.2E -09	CH_3S^+
	O_2^+	62	60	2.2E -09	$(CH_3)_2S^+$
dimethyl thioether	NO^+	62	100	2.20E -09	$(CH_3)_2S^+$
methyl mercaptan	H_3O^+	49	100	1.80E -09	$CH_4S.H+$
hydrogen sulphide	H_3O^+	35	100	1.60E -09	H_3S^+
	O_2^+	34	100	1.40E -09	H_2S^+
Amines					
dimethyl amine	H_3O^+	46	100	2.10E -09	$(CH_3)_2NH.H^+$
2-pentanamine	H_3O^+	18	60	2.7E -09	NH_4^+
trimethyl amine	H_3O^+	58	10	2.00E -09	$C_3H_8N^+$
Esters					
ethyl acetate	H_3O^+	89	100	2.90E -09	$CH_3COOC_2H_5.H^+$
	H_3O^+	107		2.90E -09	$CH_3COOC_2H_5.H^+.H_2O$
	NO^+	148	90	2.10E -09	$NO^+.CH_3COOC_2H_5$
Acids					
acetic acid	NO^+	90	100	9.0E -10	$NO^+.CH_3COOH$
	NO^+	108		9.0E -10	$NO^+.CH_3COOH.H_2O$
	O_2^+	60	50	2.3E -09	CH_3COOH
Other					
ammonia	O_2^+	17	100	2.40E -09	NH_3^+
diethyl ether	H_3O^+	75	100	2.4E -09	$C_2H_5OC_2H_5.H^+$

	NO^+	73	100	1.8E -09	$C_4H_9O^+$
	O_2^+	31	15	2.0E -09	CH_3O^+
ethylene oxide	H_3O^+	45	100	2.40E -09	$C_2H_5O^+$
	NO^+	74	100	1.00E -10	$C_2H_4O.NO^+$
pentane	O_2^+	42	40	1.6E -09	$C_3H_6^+$