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Molecular identification of the dominant microbiota and their spoilage potential of *Crangon crangon* and *Raja* sp.

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Katrien

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LIST OF NON-STANDARD ABBREVIATIONS

List of non-standard abbreviations

APC	Aerobic psychrotrophic count
ATP	Adenosine triphosphate
a_w	Water activity
BLAST	Basic local alignment search tool
bp	Basepairs
*car*A	Small subunit of the carbamoyl phosphate synthase gene
CFC	Cetrimide Fucidine Cephaloridine
cfu/g	Colony forming units / gram
CO_2	Carbon dioxide
PCR-DGGE	Denaturant gradient gel electrophoresis
DMA	Dimethylamine
DMDS	Dimethyldisulfide
DMS	Dimethylsulfide
DNA	Deoxyribonucleic acid
DVB/CAR/PDMS	divinylbenzene/carboxen/ polydimethylsiloxane
E200	Sorbic acid
E201	Sodium sorbate
E210	Benzoic acid
E211	Sodium benzoate
E223	Sodium metabisulfite
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
EU	European Union
eV	Electronvolt
GC-MS	Gas chromatography coupled to mass spectrometry
g*yr*B	DNA gyrase subunit B
H_2S	Dihydrogen sulphide
HS	Head space
HS-GC-MS	Headspace gas chromatography mass spectrometry
Hx	Hypoxanthine
IA	(Lyngby) Iron agar
ICMSF	International Commission on Microbiological Specifications for Foods
ILVO	Institute for Agriculture and Fisheries Research
Ino	Inosine
ISO	International Organization of Standardization
LAB	Lactic acid bacteria
LH	Long and Hammer medium
LOD	Limit of detection
MA	Marine agar
MAP	Modified atmosphere packaging
MIM	Multiple ion monitoring
MRD	Maximum recovery diluent

MRS	Man Rogosa Sharp medium
MSA	Metabolic spoilage association
NH_3	Ammonia
NIST	National Institute of Standards and Technology
NJ	Neighbour joining
NMKL	Nordic Comittee on Food Analysis
NPN	Non-protein nitrogen
Pa	Pascal pressure unit
PBS	Phosphate buffered saline
PCA	Plate count agar
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppb	Part per billion
PTFE	Polytetrafluoroethylene
PTV injector	Programmed temperature vaporisator injector
QIM	Quality index method
rDNA	Ribosomal deoxyribonucleic acid
REP	Repetitive extragenic palindromic
rep-PCR	Repetitive element sequence based polymerase chain reaction
RI-HPLC	Refractive index - high-performance liquid chromatography
RNA	Ribonucleic acid
*rpo*B	RNA polymerase subunit B
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SIFT-MS	Selected ion flow tube mass spectrometry
sp.	Species (singular)
SPME	Solid phase micro extraction
spp.	Species (plural)
SSO	Specific spoilage organism
SSSP	Seafood spoilage and safety predictor
STAA	Streptomycin-thallous-acetate-actidione
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TGGE	Temperature gradient gel electrophoresis
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TVB	Total volatile bases
VOC	Volatile organic compound
VRBGA	Violet red bile glucose agar
w/v	Weight/volume
x	Mean value

CHAPTER 1

INTRODUCTION, OBJECTIVES AND OUTLINE

Chapter 1. Introduction, objectives and outline

Introduction

Seafood is a healthy food product which is necessary in human diet for the uptake of omega-3-fatty acids, several vitamins and trace elements. The public awareness of this health advantage leads to an increase in the demand for especially fresh and mildly preserved seafood. However, the very rapid spoilage of seafood, which is mainly due to microbiological activity, cannot easily be reconciled with a busy life style. The microbiota on seafood is matrix-specific and depends on many factors including the intrinsic properties of the species, the environment, water temperature, area of catch, and handling and processing procedures (Jay, 1986). The unpleasant and offensive off-odours and off-flavours that lead to sensory rejection by consumers and restricted the shelf life of the seafood are produced by microbiological degradation of soluble, low molecular weight components (Gillespie and Macrae 1975; Gram and Dalgaard 2002; Gram et al. 2002; Herbert et al. 1971; Shewan and Murray 1979). The volatile organic compounds (VOCs) associated with spoilage are produced by only a fraction of the microbiota present on the seafood during storage, generally known as "specific spoilage organisms" or SSOs (Dalgaard 1995). Each fish or fishery product will have its own SSOs and the number of these will, as opposed to the total number, be related to the shelf life. Therefore it is important to identify the SSOs and observe their spoilage potential in order to ultimately advise on measures for improving the quality and shelf life.

Objectives

- **Optimisation of the detection and identification techniques** to obtain a broad view of the microbiota present on seafood during air storage on ice.

- **Implementation of the optimised techniques for the identification and characterisation of the dominant microbiota** of two Flemish fishery products, brown shrimp and ray.

Outline

In **Part I**, culture-dependent (e.g. conventional plating techniques) as well as (molecular) culture-independent techniques [e.g. polymerase chain reaction - denaturant gradient gel electrophoresis (PCR-DGGE)] were optimised. **Chapter 3** outlines the limitations of the

growth media currently used by food business operators, government agencies, retailers, distribution quality laboratories, and researchers.

The identification and characterisation of the dominant microbiota of brown shrimp and ray, targeted here were used to achieve more knowledge in the spoilage process of these fishery products, in order to achieve a better estimation of their quality and, where possible, to obtain an extension of shelf life (Part II and Part III). These fishery products were chosen based on their special characteristics correlated to a high sensitivity to spoilage, e.g. a very high number of non-protein nitrogenous compounds (NPN fraction) compared to other seafood, which are easily metabolised by microorganisms (Liston 1980). In **Part II,** the **SSOs of brown shrimp** (*Crangon crangon*) are described. Brown shrimp is a typical product of the Belgian fishery and recently its formulation without preservatives received the local quality label, "Purus". It is exclusively caught in the North Sea and prepared by Flemish fishermen, predominantly in a traditional way. **Chapter 4** outlines the dominant microbiota of brown **unpeeled and peeled preservative-free shrimp during air storage** under different temperature conditions. Special attention was given to the molecular identification of each isolate, since **accurate species identification** is required to link particular species to increased spoilage risks**.** Sequencing of household genes were used for species identification. The **spoilage potential of the dominant isolates by their volatile organic compound (VOC) production** on sterile shrimp was studied in **chapter 5** via gas chromatography coupled to mass spectrometry (GC-MS) and selected ion flow tube mass spectrometry (SIFT-MS) analysis. **Part III** deals with the spoilage microbiota of ray (*Raja* sp.) during ice storage. Ray is a commercial elasmobranch fish species important for the Belgian market. Elasmobranch fish species are especially known for their short shelf life due to rapid ammonia production. In **Chapter 6,** the dominant microbiota of ray and their spoilage potential was studied under several conditions in order to postpone ammonia production.

In summary, this thesis contributes to the optimisation of detection and identification techniques used to observe and reveal the SSOs of seafood. These techniques were applied to identify and characterise SSOs of brown shrimp (Part II) and ray (Part III).

The research outline of this PhD study is presented in Figure 1.1.

Chapter 2. Seafood spoilage and quality analysis techniques: a literature overview

PART I. SEAFOOD QUALITY ASSESSMENTS: OPTIMISATION OF TRADITIONAL DETECTION TECHNIQUES

Chapter 3. Seafood quality analysis: molecular identification of dominant microbiota after ice storage on several general growth media

IMPLEMENTATION OF TECHNIQUES: IDENTIFICATION AND CHARACTERISATION OF THE DOMINANT MICROBIOTA

PART II. BROWN SHRIMP (*CRANGON CRANGON*): THE DOMINANT MICROBIOTA AND THEIR SPOILAGE POTENTIAL

Chapter 4. Molecular identification of the microbiota of peeled and unpeeled brown shrimp (*Crangon crangon*) during storage on ice and at 7.5°C

Chapter 5. Volatile compounds associated with *Psychrobacter* spp. and *Pseudoalteromonas* spp., the dominant microbiota of brown shrimp (*Crangon crangon*) during aerobic storage

PART III. RAY (*RAJA SP.*): THE DOMINANT MICROBIOTA AND THEIR SPOILAGE POTENTIAL

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

CHAPTER 7. GENERAL CONCLUSIONS, RECOMMENDATIONS AND PERSPECTIVES

Fig. 1.1. Research outline of this PhD study

CHAPTER 2

SEAFOOD SPOILAGE AND QUALITY ANALYSIS TECHNIQUES: A LITERATURE OVERVIEW

Chapter 2. Seafood spoilage and quality analysis techniques: A literature overview

Shortly after capture, death of seafood occurs. Upon death, physical and chemical changes caused by enzymes and microorganisms start to occur. Post mortem changes in seafood tissues will take place in three stages: (1) *rigor mortis* and slime secretion (for fish), (2) autolysis through enzymatic decomposition of tissues (biochemical changes) and (3) microbiological spoilage (Sen 2005).

2.1. Microbiological and biochemical changes after catch

2.1.1. Rigor mortis

The *rigor mortis* phase that occurs shortly after death, is caused by a series of changes due to the ceasing of respiration and anabolic processes. After death, the remaining oxygen will be depleted by ATP production. Anaerobic breakdown of glucose takes place, whereas ATP synthesis stops and hydrolysis starts (Fig. 2.1) (Sen 2005). The *rigor mortis* state is well

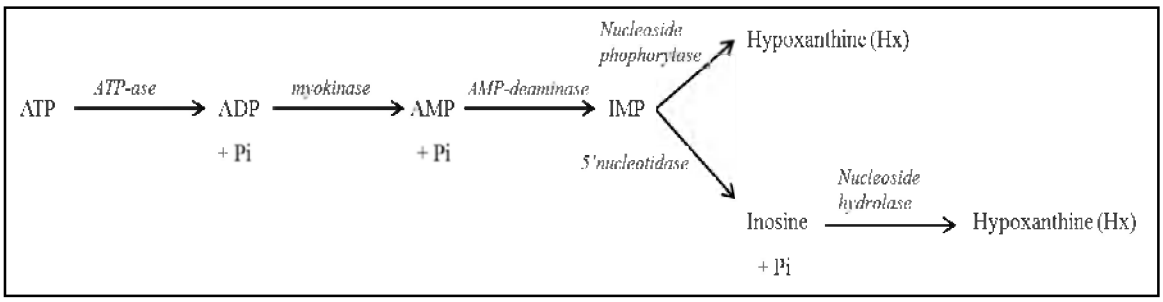

Fig. 2. 1. Break down of ATP to hypoxanthine

understood in vertebrate muscle, but has not been investigated in many crustaceans, where for some it probably seems to be absent.

The stiffening of the muscles during *rigor mortis* is caused by ATP depletion. ATP is needed in muscle contraction and relaxation (Fig. 2.2). It supplies energy for the contraction and removal of calcium ions via a calcium pump, which breaks down the actomyosin complex, leaving the muscle ready for a further contraction. Upon death, ATP levels drop and calcium ions leak forming actomyosin. However, there is insufficient ATP for the calcium pump, so the actomyosin complex remains unbroken and the muscle is in a continual state of rigidness (Nicholls and Ferguson 2002).

Rigor usually starts at the tail. Seafood remains rigid for a period which can vary from an hour to three days, depending on a number of factors. The onset of *rigor mortis* depends on the seafood, the catching technique (rough handling and stress can shorten the time of occurrence and duration of *rigor mortis*) and the temperature of the seafood (Kiessling et al. 2006; Skjervold et al. 2001).

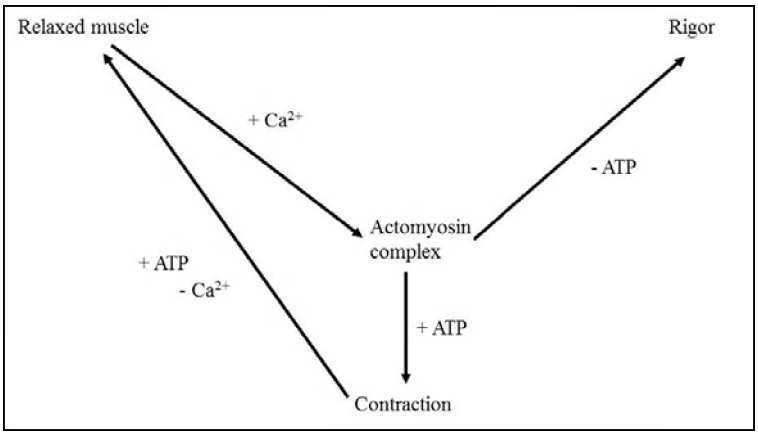

Fig. 2. 2. Scheme of the contraction and relaxation of muscle in the presence and absence of ATP energy supply

The onset is correlated to the glycogen reserves of the muscle prior to death, which is directly related to the action and stress the fish has undergone prior to capture and death. The lower the glycogen reserve, the faster the onset; in active seafood, *rigor mortis* therefore proceeds very quickly (Skjervold et al. 2001). The higher the internal temperature, the sooner *rigor mortis* starts and the faster it ceases due to the faster enzymatic reactions (Kiessling et al. 2006). Usually, the later *rigor mortis* begins and the longer it lasts, the longer is the storage life of the seafood. When muscles begin to soften and become limp again, the animal has passed through *rigor* and the muscle is in *post-rigor* condition (Stroud 2001).

2.1.2. Seafood spoilage by autolysis and biochemical changes during aerobic storage

Seafood spoilage itself starts due to two causes: autolysis and microbiological activities (see 2.1.4.).

Autolysis results in the breaking down of proteins, nucleotides, and sugars and the oxidation of fatty acids. This leads to a release of free bases and a pH drop. These make the seafood smelly, rancid, and tough. Tissue enzymes in the muscle break down desirable compounds into tasteless or bitter ones, whilst gut enzymes attack the internal organs and allow microbiological penetration into the flesh. Initially, the flesh is considered sterile; only the outer surface and the intestines contain bacteria (Shewan 1962). Once autolysis begins, the

bacteria are able to enter the flesh, after which they rapidly multiply and decompose the muscle (Mukundan et al. 1986).

During autolysis, several biochemical changes occur (Mukundan et al. 1986). These changes, as described below, are often used as quality indicators (see 2.2.3.).

- Some factors, such as slow freezing and variability of storage conditions will cause protein denaturation. A denatured enzyme will lose its water-holding capacity and will cause excessive dripping upon thawing (drip-thaw). The physical appearance of the flesh will change and it will become fibrous and tasteless.

- Living seafood has a nearly neutral pH. Autolysis will decrease this pH due to the anaerobic break down of glycogen via glycolysis to lactic acid. The concentration of lactic acid depends on the muscles' glycogen reserves prior to death. This decline in pH (below 6.6) will affect the quality of the tissues, with a more firm flesh and the enhancement of drip.

- The autolytic production of volatile bases. The total volatile bases (TVB) value is the total amount of volatile nitrogen compounds produced during storage. These nitrogen compounds such as trimethylamine (TMA) are produced due to autolytical, but especially due to microbiological activity (see 2.1.4.2.).

- Lipid hydrolysis and oxidation are two major deteriorative changes and comprise: (1) the enzymatic hydrolysis of lipids to fatty acids and glycerol and (2) the oxidation of fatty acids yielding rancid odours and aromas, which are a major problem encountered in fish storage.

- Nucleotide [adenosine triphosphate (ATP)] break down to hypoxanthine (Hx) (Fig. 2.1.). This biochemical change is also used in the "freshness" index (K-value), as described in 2.2.3.

2.1.3. Biochemical changes under aerobic conditions

Biochemical composition of seafood

In general, seafood flesh is composed of 66 to 81% water, 16 to 21% proteins, 0.2 to 25% lipids and 0.1 to 3% minerals (Love 1970; Stansby 1962). The chemical composition in seafood muscles depends on the type of seafood and also fluctuates depending on size,

season, fishing grounds, feed intake (diet), migratory swimming, and sexual changes in connection with spawning (Shewan 1961).

Fresh and mildly preserved seafood are prone to rapid spoilage mainly due to their high number of non-protein nitrogenous compounds (NPN fraction) which are easily metabolised by microorganisms (Drosinos and Board 1994; Gill 1976; Liston 1980). This NPN fraction contains the water-soluble, low molecular weight, nitrogen-containing compounds of non-protein nature. The major components in this fraction (Fig 2.3) are volatile bases such as ammonia and trimethylamineoxide (TMAO), creatine, free amino acids, nucleotides and purine bases, and in case of cartilaginous fish, urea (Brown 1986; Huss 1995; Jay 1986; Shewan 1961).

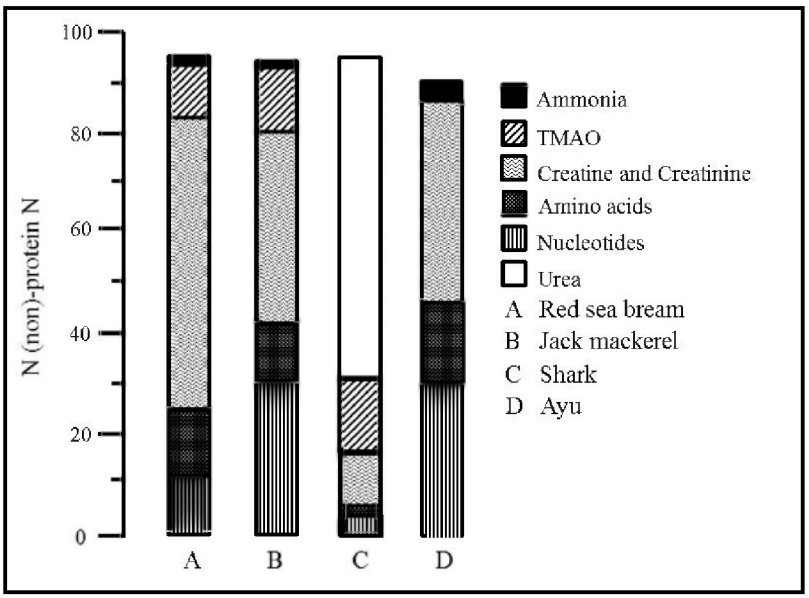

Fig. 2. 3. Distribution of non-protein nitrogen in fish muscles of two marine bony fish (A, B), an elasmobranch (C), and a freshwater fish (D) (Konosu and Yamaguchi, 1982; Suyama et al., 1977).

Biochemical changes during storage

When seafood is consumed, the quality is observed based on the sensory characteristics. These characters may be grouped as appearance, odour, flavour and texture. The odour of freshly caught seafood is described as a weak odour typical of the sea or sea weedy (Triqui 2006). If seafood is held on ice from the time of catch, it retains its high quality for a short period of time depending on their characteristics. However, prolonged storage or inappropriate storage (e.g. at ambient temperature) may lead to the development of an undesirable "fishy" odour. During spoilage, microbiological degradation of soluble, low molecular weight components results in the formation of volatile metabolites such as alcohols, ketones, sulphur compounds [e.g. dihydrogen sulphide (H_2S), dimethyl sulphide (DMS) and dimethyl disulphide (DMDS)], amines [e.g. TMA, dimethyl amine (DMA)], esters, aldehydes and organic acids (Gram and Dalgaard 2002; Gram et al. 2002).

These metabolites are responsible for the unpleasant and offensive off-odours and off-flavours that lead to sensory rejection and shorten the shelf life of the seafood (Gillespie and Macrae 1975;Herbert et al. 1971;Shewan and Murray 1979). Not all microorganisms on seafood are able to produce those metabolites, but mainly the SSOs. As shown in Table 2.1 for three major spoilage organisms, the produced volatiles are species-specific. In general, the capable microorganisms produce volatile metabolites in concentrations important for spoilage once they have reached a total count of 10^8-10^9 cfu/g (Dalgaard 1995b; Gram et al. 2002). Next to the potential of the microorganisms to form metabolites associated with spoilage, also the substrates for production need to be present in the matrix. The possible substrates for the production of the most important spoilage volatiles are listed in Table 2.2.

Especially the presence of TMA is considered a very important spoilage indicator for marine seafood, since this compound is the cause of the fishy smell that occurs during spoilage. The precursor, TMAO is typical for several marine seafood species. The TMAO concentration of seafood depends mainly on the depth that they live; the deeper the water, the higher the TMAO level in the muscles (Kelly and Yancey 1999). TMA is produced particularly by the decomposition of TMAO by *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Vibrio* spp., *Aeromonas* spp. and psychrotolerant *Enterobacteriaceae* (Fig. 2.4.).

These bacteria all contain the enzyme TMAO reductase that catalyses the reduction of TMAO. The compound is produced in much higher amounts when fish is stored under low oxygen conditions, since *P. phosphoreum* and *S. putrefaciens* use TMAO instead of oxygen

as final electron acceptor in their metabolism (Boskou and Debevere 1997; Dalgaard et al. 1993; Gram and Dalgaard 2002). This makes TMA production a useful quality index method, but only during the middle and the last stages of spoilage when bacteria have invaded the fish flesh and reduce TMAO. TMA can be further converted in the muscle tissue into DMA and formaldehyde by enzyme action during frozen storage.

Table 2. 1. The spoilage potential of three important spoilers based on their ability to produce volatiles

Microorganism	Potential to produce spoilage volatiles	References
Photobacterium phosphoreum	Alcohols	(Chai et al. 1968; Dalgaard 2006; Olafsdottir et al. 2005b)
	Amines E.g. TMA	(Chai et al. 1968; Dalgaard 2006; Olafsdottir et al. 2005b)
	Hypoxanthine	(Chai et al. 1968; Dalgaard 2006; Olafsdottir et al. 2005b)
	Ketones E.g. acetone, acetoin	(Chai et al. 1968; Dalgaard 2006; Nemecek-Marshall et al. 1999; Olafsdottir et al. 2005b)
***Pseudomonas* spp.**	Alcohols E.g. methanol, ethanol	(Chinivasagam et al. 1998; Freeman et al. 1976)
	Amines E.g. TMA	(Nychas et al. 2007)
	Ammonia	(Reynisson et al. 2009; Schmitt and Schmidtlorenz 1992)
	Esters E.g. ethyl acetate	(Edwards et al. 1987; Freeman et al. 1976; Reynisson et al. 2009)
	Ketones E.g. acetone, 2-pentanone	(Chinivasagam et al. 1998; Pittard et al. 1982)
	Sulphur compounds E.g. DMS, DMDS, methylmercaptan	(Freeman et al. 1976; Nychas et al. 2007; Pittard et al. 1982; Reynisson et al. 2009)
Shewanella putrefaciens	Alcohols E.g. 2,3-butanediol, 2-pentanol	(Chinivasagam et al. 1998; Joffraud et al. 2001)
	Amines E.g. TMA	(Joffraud et al. 2001; Reynisson et al. 2009)
	Esters E.g. ethyl esters	(McMeekin 1982)
	Hypoxanthine	(Reynisson et al. 2009)
	Ketones	(Chinivasagam et al. 1998)
	Sulphur compounds E.g. H_2S, methylmercaptan, DMDS	(Freeman et al. 1976; Joffraud et al. 2001; Reynisson et al. 2009)

The association between seafood spoilage and volatile sulphides is well known (Varlet and Fernandez 2010). Due to the low odorant thresholds, those compounds can rapidly give a putrid odour to seafood (Chung and Cadwallader 1993). The odours corresponding to the production of sulphur compounds are described as rotten vegetable (e.g. cabbage) smell, onion or garlic odour, etc. (Varlet and Fernandez 2010). Sulphur-containing amino acids such as methionine and cysteine are the key components in the production of sulphur-containing volatiles in seafood (Varlet and Fernandez 2010). The measurement of sulphur-containing

Table 2. 2. Possible substrates for the production of the most important volatile organic compounds (off-odours) by microbiological activity in (sea) food (Herbert and Shewan 1976; Nychas et al. 2007)

Volatile organic compound	Possible precursor
Sulphides	
Dimethylthioether	Methanethiol, methionine
Dimethyldisulfide	Methionine
Methyl mercaptan	Methionine
Sulphur hydride	Cystine, cysteine
Other	
Ammonia	Amino acids
Trimethylamine	Trimethylamine oxide

volatiles is therefore a reliable indicator for the monitoring of seafood quality (Varlet and Fernandez 2010).

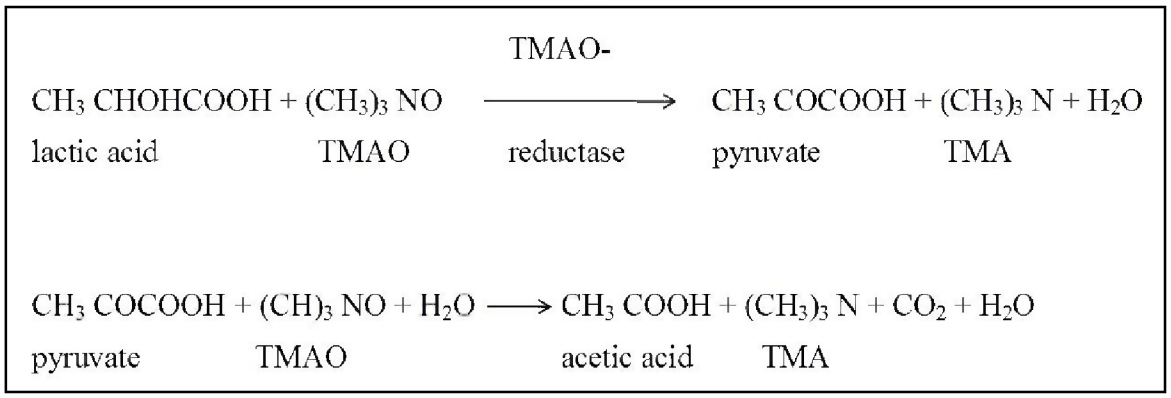

Fig. 2. 4. Reduction of TMAO to TMA by TMAO reductase.

The production of these off-odours depends not only on the intrinsic characteristics of the seafood species (e.g. chemical composition), but also on the extrinsic parameters such as the storage temperature and environment (Olafsdottir et al. 2006a). It should also be noted that chemical changes (e.g. the production of off-odours) occurring in naturally contaminated seafood differ significantly from those on sterile muscle tissue inoculated with spoilage organisms (Koutsoumanis and Nychas 1999). Single strains and mixtures of microorganisms will give different outcomes due to microbiological interaction (see 2.1.3.) (Joffraud et al. 2001).

2.1.4. Microbiological spoilage of fresh seafood

Seafood is very sensitive to spoilage. The main factors limiting shelf life are microbiological activities. Microbiological spoilage can be manifested in visible growth, textural changes or off-odours and off-flavours (Gram and Dalgaard, 2002).

The initial microbiota of marine seafood

Microbiological growth occurs immediately after *rigor mortis*, microbiological spoilage however does not. In general, seafood is contaminated with a wide range of microorganisms which occur naturally in the surrounding environment. The degree of microbiological contamination depends on several environmental factors such as the water temperature, salt content, the season of catch, fishing method, and the microbiological quality of the water, but also on species-specific factors, including the type of seafood and their diet (Feldhusen 2000; Gram et al. 1990; Grigorakis et al. 2003; Huss 1995; Jay 1986; Kaspar and Tamplin 1993; Tzikas et al. 2007; Zaballos et al. 2006).

Seafood from cold and temperate waters contains mainly psychrotrophic Gram-negative, rod-shaped, strictly aerobic or facultatively anaerobic microorganisms of the genera *Aeromonas*, *Pseudomonas*, *Moraxella/Acinetobacter*, *Shewanella*, or *Flavobacterium*, or from the family of *Vibrionaceae* (Liston 1980). Gram-positive bacteria, such as *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus*, and coryneforms may occur, albeit to a lesser extent (Elotmani et al. 2004; Gennari et al. 1999). Although it is said that seasonal differences in the microbiological load are present, significant differences in species variation have not been observed (Elotmani et al. 2004). Also, the influence of the water temperature on the species variation is disputed. Shewan (1977) reported that seafood from tropical waters contain more mesophilic Gram-positive microorganisms such as *Bacillus* and *Micrococcus*. Several other authors found a microbiological contamination similar to those of temperate waters, but often with a slightly higher contamination degree of Gram-positives and enteric bacteria (Gram et al. 1990; Jay 1986; Liston 1980).

Influence of handling and processing on the microbiota

Not only environmental features, but also (early) handling procedures [e.g. gutting, rinsing with seawater (see 2.3.1) and icing], as well as other processing procedures and storage conditions [e.g. filleting and packaging (see further)] are an important source of microbiological contamination and population shifts (Bagge-Ravn et al. 2003; Feldhusen 2000; Huss 1995; Jay 1986; Nychas et al. 2007; Reynisson et al. 2009; Shewan and Georgala 1957; Valdimarsson et al. 1998; Zapatka and Bartolomeo 1973).

One of the most important procedures to deliver high-quality seafood is immediate icing (Akankwasa 1998). This is beneficial for the onset of *rigor mortis*, but also much lower counts of microorganisms are found when the seafood is kept on ice. Thereby, different ways

of icing (e.g. flake ice or slurry ice) are often compared and often a difference in the general microbiological quality of the seafood is observed (Rodriguez et al. 2005). For many food products, including seafood, superchilling results in better quality when compared to conventional chilling (Duun and Rustad 2008; Fagan et al. 2004; Wang et al. 2008). When superchilled, food products are stored between the freezing point of the products and 1 to 2°C below this. The surrounding temperature is set below the initial freezing point of the food, which is between -0.5°C and -2.8°C (Fennema et al. 1973) and depending on the method used, some ice is formed in the outer few millimeters. This is in contrast to conventional chilling on ice where the surface of the fish, when in direct contact with the ice, is 0 ± 0.5°C. When the ice does not make contact with the seafood, the temperature will be slightly higher. Superchilling can be performed in several ways, using crushed melting ice and additional refrigeration or by using cold air tunnels (most effective). This superchilling may delay the microbiological growth of microorganisms such as *Photobacterium phosphoreum* while other dihydrogen sulphide (H_2S)-producing bacteria, most likely *Shewanella* spp., are not affected (Olafsdottir et al. 2006b).

Specific spoilage organisms (SSOs) of marine seafood

Research has revealed that the total number of microbiota on seafood is not responsible for spoilage, but rather only a small fraction of the microorganisms, the SSOs (Dalgaard 1995b). SSOs must be enumerated and eventually identified for quality control or determination of the remaining shelf life (Dalgaard 1995a). SSOs are specific to each seafood species and storage conditions (Table 2.3. and 2.4.), and are still unknown for most seafood. Microorganisms such as *Pseudomonas, Shewanella putrefaciens*, *Shewanella baltica*, *Photobacterium phosphoreum* and *Brochothrix thermosphacta* are commonly known SSOs of seafood during cold storage (Dalgaard 1995b; Dalgaard et al. 1997; Emborg et al. 2002; Gram et al. 1990; Gram and Huss 1996; Lauzon et al. 2009; Mejlholm et al. 2005; Olafsdottir et al. 2006a; 2006b; Paarup et al. 2002; Vogel et al. 2005). The microbiological activity and especially the formation of volatiles (see 2.1.3.) such as TMA, ammonium and H_2S of SSOs such as *Shewanella* sp., *Photobacterium phosphoreum* and *Pseudomonas* sp. (Dalgaard 1995b; Koutsoumanis and Nychas 1999; Tryfinopoulou et al. 2002; Vogel et al. 2005), contribute to the off-flavours and taste associated with spoiled seafood. A close relationship between the log number of the SSOs and the shelf life may be expected (Dalgaard 1995a).

Population shifts during different storage conditions

During storage, the composition of the initial microbiological population may shift (Reynisson et al. 2009; Shewan and Georgala 1957). In general, seafood is kept in chilled storage and preferable on ice. This allows the psychrotolerants (e.g. *Pseudomonas* spp.), which grow optimal at chilled conditions, to dominate the microbiota during storage (Castell and Mapplebeck 1952; Moore et al. 2006; Tryfinopoulou et al. 2002). Air stored chilled or iced conditions often promote the growth of *Pseudomonas* spp., *Shewanella putrefaciens*, and *Psychrobacter immobilis* (Gennari et al. 1999). Temperature fluctuations will influence the microbiota during storage. For instance, abusive temperature can create an optimal environment for strong microbiological spoilers such as *Photobacterium phosphoreum* or other microorganisms which are able to produce biogenic amines (Olafsdottir et al. 2006b).

The microbiota and shelf life of seafood will also be influenced by storage conditions (Table 2.3) such as aerobic storage, vacuum or modified atmosphere packaging (MAP) (Table 2.4) (Poli et al. 2006). MAP packaging will extend the shelf life of fresh fish significantly due to its inhibitory effect on microbiological growth. It will also reduce certain undesirable physiological, chemical/biochemical and physical changes in foods (Floros and Matsos 2005).

Table 2.3. Seafood SSOs for fresh seafood stored under different conditions

Product	SSO	References
Fresh chilled seafood	*Shewanella putrefaciens*, *Pseudomonas* spp.	(Gram and Huss 1996;Molin and Stenström 1984)
Fresh chilled seafood vacuum or MAP packed	Lactic acid bacteria (LAB), *Photobacterium phosphoreum*, *Brochothrix thermosphacta*	(Dalgaard et al. 1993;Gram and Huss 1996;Jeppesen and Huss 1993;Rudi et al. 2004)
Fresh seafood stored at >10°C	*Vibrionaceae*, *Enterobacteriaceae*	(Lindberg et al. 1998;Molin and Stenström 1984)
Cooked shrimp, MAP stored	LAB, *Brochothrix thermosphacta*	(Dalgaard et al. 2003;Dalgaard and Jorgensen 2000)

The best known SSO of fresh MAP packed seafood is *Photobacterium phosphoreum*. (Dalgaard 1995b; Dalgaard et al. 1993; 1997). Based on the gas mixture (Table 2.4), MAP packed fresh seafood, may reduce the growth of several microorganisms important for the production of off-odours and off-flavours such as *Pseudomonas* spp., *Aeromonas* spp., and *Shewanella putrefaciens*. These are all microorganisms which are able to outcompete other microbiological groups/microorganisms when air stored (Table 2.3) (Gennari et al. 1999; Gram and Huss 1996; Hansen et al. 2007; Lalitha et al. 2005; Leroi 2010; Poli et al. 2006).

Table 2.4. Effect of gas composition on the specific spoilage organisms dominating fresh fish stored at 0 to 4°C under different gas atmospheres (Gram and Huss 1996; Gram and Huss 2000; Huss et al. 1997; Lalitha et al. 2005; Poli et al. 2006)

Gas composition	Microorganisms in fish
Air	*Shewanella putrefaciens*, *Pseudomonas* spp.
>50% CO_2 with O_2	*Brochothrix thermosphacta*, *S. putrefaciens*
50% CO_2	*Photobacterium phosphoreum*, lactic acid bacteria (LAB)
50% CO_2 with O_2	*P. phosphoreum*, LAB, *B. thermosphacta*
100% CO_2	LAB
Vacuum packed	*Pseudomonas* spp., *P. phosphoreum*

Interactions among spoilage bacteria

The microbiological association and subsequent chemical changes that occur during spoilage depend on environmental conditions but also microbiological interactions are of great importance (Gram and Melchiorsen 1996; Gram et al. 2002). Several types of interactions between food spoilage bacteria can be observed, such as antagonism and metabiosis (inter-dependency) (Gram et al. 2002).

The promotion of changes in the environmental conditions, e.g. decreasing pH, can create a selective advantage. Competition for nutrients, such as minerals, proteins and amino acids, may also select for the microorganisms best capable of utilizing the limiting compound. An important parameter in fish is the limiting concentration of iron (Gram 1994). Several bacteria growing on seafood produce siderophores (Gram and Melchiorsen 1996) that are only induced when the iron concentration is low. Especially *Pseudomonas* spp. isolated from fish are prominent producers of siderophores (Gram 1993). When *Pseudomonas* spp. are grown in co-culture with *Shewanella putrefaciens*, the growth of the latter is inhibited. Other examples comprise certain Gram-negative microorganisms which produce NH_3 and TMA, toxic to a number of other bacteria and sometimes to the producing organism itself. Also lactic acid bacteria (LAB) are able to outcompete other microorganisms (e.g. *Listeria monocytogenes*) for essential nutrients and to decrease the pH via the production of lactic acid or the production of antimicrobiological peptides (bacteriocins) (Adams and Nicolaides 1997; Buchanan and Bagi 1997; Nilsson et al. 1999). Therefore, more and more research focusses on a possible role as natural preservative of LAB in seafood (Leroi 2010).

Next to antagonism, innumerable ways of inter-dependency (metabiosis) exist between different organisms, such as the production of off-odours by a mixture of microorganism and

not by the strains individually. Joffraud et al. (2001) have observed that the separate inoculation of three Gram-negative bacteria (S*hewanella*, *Photobacterium* and *Aeromonas*) did not cause spoilage in cold-smoked salmon. However, when one of those was co-inoculated with *B. thermosphacta* and *Carnobacterium maltaromaticum* spoilage off-odours were produced. Therefore, Jorgensen et al. (2000) created the term "metabolic spoilage association" (MSA) to describe situations where two or more microbiological species contribute to spoilage through metabolite or nutrient exchange.

2.2. Quality assessments of seafood in the industry

The quality can be explained by several sensory, microbiological, biochemical and physical parameters (Fig. 2.5).

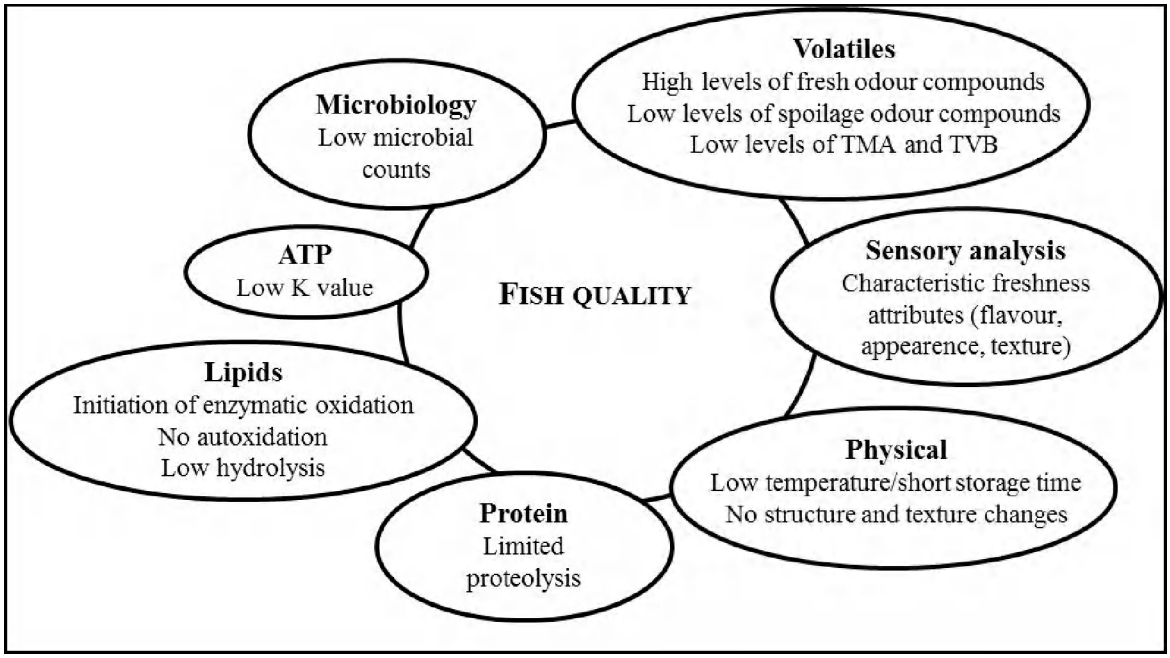

Fig. 2. 5. Summary of the various approaches used to evaluate fish quality according to Olafsdottir et al. (1997). The K value is defined as the ratio of the sum of inosine and hypoxanthine concentrations to the total concentration of adenosine triphosphate (ATP) catabolites. TMA: trimethylamine; TVB: total volatile bases.

2.1.1. Sensorial evaluation of the seafood quality

The sensorial evaluation of seafood is defined as the discipline used to evoke, measure, analyse and interpret characteristics of the food as perceived by the senses of sight, smell, taste, touch and hearing (Olafsdottir et al. 1997). When seafood deteriorates, its characteristic appearance, odour, taste, and texture change. Therefore, the quality of seafood is determined by the sensorial evaluation based on a number of external features such as skin, eyes, slime, gills, incision, texture, and taste by highly specialized trained assessors. In Europe, the most commonly used method for the quality assessment of raw seafood is the European Union

(EU) scheme (Anonymous 1996). The EU scheme uses three different classes of freshness: E(xtra), A and B. The E class stands for the highest quality, whereas seafood below the B class quality is not suitable for consumption. This scheme does not take into account differences between species since only general parameters are used (Olafsdottir et al. 1997). An alternative scaling method is the quality index method (QIM). This method has been suggested since it uses precise, objective and independent descriptions of the individual grades for each species individually (Larsen et al. 1992). The QIM is based on the significant sensorial parameters for raw seafood. The scores for all of the characteristics are then added to give an overall sensorial score, the quality index, which can be used to predict storage life. The QIM score will linearly increase with the storage time on ice. Since the potential storage time on ice is known for each product, also the remaining shelf life can be calculated based on the QIM score. Bekaert at al. (2007) have developed QIM parameters for several North Sea fish species and other fishery products.

2.2.2. Evaluation of the microbiological quality of seafood

The main factor limiting shelf life of fresh seafood is microbiological activity. In order to measure the microbiological quality of seafood, several parameters are observed (Table 2.5). When seafood reaches a microbiological contamination level associated to the end of shelf life, the seafood will be considered not appropriate for consumption. As described in chapter 3, quality control and potential shelf life of fish is currently still often estimated based on the total aerobic psychotropic count (APC). As recommended by the International Organization for Standardization (ISO), the psychotropic enumeration of microorganisms on food, including fish, must be performed on plate count agar (PCA) without addition of extra salt or minerals. It is assumed that an APC of 10^7-10^8 cfu/g is reached when spoilage becomes organoleptically detectable (Liston, 1980). Standards, guidelines, and specifications as part of purchase agreements of chilled fish quality only accept a much lower APC of 10^6 cfu/g for human consumption (Anonymous, 1986). Next to the APC, the presence of other microorganisms e.g. LAB and hygiene indicators and pathogens, yeasts and mould are observed [Table 2.5. (raw seafood) and Table 2.6. (cooked seafood)].

More recently it was shown that the correlation between the SSOs such as *Shewanella putrefaciens* and *Photobacterium phosphoreum* and freshness is much higher than between

Table 2. 1. Summary of the microbiological parameters and values in relation to the microbiological quality of raw seafood (Uyttendaele et al. 2010). * = legal limit.

Parameter	Goal (cfu/g)	Tolerance (cfu/g)	End of shelf life (cfu/g)
Total aerobic psychotropic count	10^5	10^6	10^7
Psychotropic lactic acid bacteria	10^2	10^3	10^7
Yeasts	10^2	10^3	10^5
Moulds	10^2	10^3	No visual growth
Escherichia coli	10^2	10^3	10^3
Coagulase positive staphylococci	10^2	10^3	10^3
Sulphite reducing bacteria	10^2	10^3	10^5
Salmonella spp.	Absent in 25g	Absent in 25g	Absent in 25g
*Listeria monocytogenes**	Absent in 25g	Absent in 25g	10^2

APC and freshness (Chai et al. 1968;Gram et al. 1987;Herbert et al. 1971;Jorgensen et al. 1988). The presence of these SSOs at the beginning of storage life, in combination with the storage conditions can be used to provide accurate information of the remaining shelf life and the safety (ability of pathogens to grow) of the seafood by mathematical models such as SSSP (seafood spoilage and safety predictor) (Dalgaard et al. 2002).

Table 2. 2. Summary of the microbiological parameters and values in relation to the microbiological quality of cooked seafood with post-cooking contamination (Uyttendaele et al. 2010). * = legal limit.

Parameter	Goal (cfu/g)	Tolerance (cfu/g)	End of shelf life (cfu/g)
Total aerobic psychotropic count	10^3	10^5	10^6
Total anaerobic psychotropic count	10^3	10^4	10^6
Psychotropic lactic acid bacteria	10^2	10^3	10^7
Yeasts	10^2	10^3	10^5
Moulds	10^2	10^3	No visual growth
Enterobacteriaceae	$5x\ 10^1$	$5x\ 10^2$	n.a.
Escherichia coli	$<10^1$	$5x\ 10^1$	$5x\ 10^1$
E. coli *	10^0	10^1	10^1
Coagulase positive staphylococci*	10^2	10^3	10^3
Sulphite reducing bacteria	10^2	10^3	10^5
Salmonella spp.*	Absent in 25g	Absent in 25g	Absent in 25g
Listeria monocytogenes *	Absent in 25g	Absent in 25g	10^2
*Bacillus cereus**	10^2	10^3	10^5

2.2.1. Evaluation of the quality of seafood based on biochemical methods

Odour is one of the most important parameters to observe seafood quality. Therefore, the measurement of volatile compounds can be used to monitor quality. The volatile compounds characterizing spoilage can be characterized as sweet, fruity, ammoniacal, sulphur and putrid (Olafsdottir et al. 1997) and comprise alcohols, ketones, sulphur compounds (e.g. H_2S, DMS and DMDS), amines (e.g. TMA, DMA), esters, aldehydes and organic acids (Gram and Dalgaard 2002; Gram et al. 2002). Classical chemical methods to observe seafood quality are mainly the analysis of TMA, TVB, and the K-value (Olafsdottir et al. 1997). As described in 2.1.4., TMA is considered a very important spoilage indicator for marine seafood, since this compound is the cause of the fishy smell that occurs during spoilage.

TVB analysis includes the measurements of TMA, DMA, ammonia and other volatile base nitrogen compounds produced by microbiological degradation of proteins and amino acids (Gram and Huss 1996; Olafsdottir et al. 1997).

The K-value or "freshness" index concerns the nucleotide degradation to hypoxanthine. The production of hypoxanthine is in many seafood species paralleled by the development of TMA (Jorgensen et al. 1988). Hypoxanthine can be formed by autolytic decomposition of nucleotides (see 2.1.1. and 2.1.2. and Fig 2.1), but also by microbiological activity, which displays a higher formation. Several spoilage bacteria produce hypoxanthine from inosine or inosine monophosphate, including *Pseudomonas* spp., *S. putrefaciens* and *P. phosphoreum* (Jorgensen and Huss 1989; Surette et al. 1988; Van Spreekens 1977). The K-value is determined by the formula in Figure 2.7 (Saito et al. 1959):

$$K\% = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]}$$

Fig. 2. 6. The K-value formula by Saito et al. (1959), where [ATP], [ADP], [AMP], [IMP], [Ino] and [Hx] represent the concentrations of these compounds in fish muscle measured at various times during chilled storage. Ino= inosine, Hx= hypoxanthine, ATP= adenosinetrifosfaat, ADP= adenosinedifosfaat, AMP= adenosinemonofosfaat, and IMP= inosine monophosphate.

More recently, a rapid assessment of volatile compounds using gas sensors (electronic noses) is of increasing interest (Haugen et al. 2006;Haugen and Kvaal 1998;Haugen and Undeland 2003;Jonsdottir et al. 2008;Olafsdottir et al. 2005a).

2.2.2. Evaluation of the quality of seafood based on physical methods

Physical changes in seafood are mainly related to structure and colour. To determine structural changes several techniques can be used in the industry such as texturometers, macroscopy, and microscopy (Olafsdottir et al. 1997). Changes in freshness can also be measured by the dielectrical properties of the muscle. As seafood spoil, microbiological and enzyme activity within the muscle structure breaks down the cellular structure in a slow and systematic way. Dielectric changes are caused which can be observed with, for instance, the Torry meter or the Fishtester. These methods can however not be used for thawed seafood or seafood stored in chilled seawater, and also the use for fish fillets is limited since a water-ice content with high salt content and mechanically damaged fish may cause erroneous results. The advantage of this method is the immediate response, the possibility for field use, and the fact that there is no need for experienced personnel (Olafsdottir et al. 1997).

Changes in seafood quality can also be correlated with colour changes. For instance, fluorescent spectra can be used to determine whether seafood has been frozen and to determine the time of storage since the intensity decreases with time storage on ice (Olafsdottir et al., 1997).

2.3. Target species

2.3.1. Brown shrimp (*Crangon crangon*)

Biology, fishery and the importance of the species in Belgium

About 1950 different families of shrimp have been described worldwide, of which 330 families are used for consumption. Brown shrimp (*Crangon crangon*) belong to the family of the *Crangonidae* or sand shrimp, which can be found in cold to average waters in the Northern hemisphere. These are highly reproductive crustaceans with a short life time of about one to possible three years. The distribution ranges from the North Atlantic (Norway, Iceland) to North African waters and the Mediterranean. However, only shallow coastal waters such as the southern North Sea give abundance rates that form the basis of an intensive fishery (Anonymous, 2010). Especially the countries in North-West Europe intensively catch North sea shrimp, with an annual supply of maximal 3500 tonnes, which places the brown shrimp in the top 10 of the most important commercial shrimp species. The Netherlands, Denmark, and Germany represent about 95% of the total North Sea production. Over 80% of the EU market is controlled by two Dutch companies, Heiploeg and Klaas Puul, which buy about 30000 tonnes of brown shrimp a year. Brown shrimp is considered as a speciality in

Belgium, which makes Belgium the main consumer market with more than half of the total EU market (1500 tonnes) for brown shrimp (Anonymous, 2010). Although Belgium has a smaller fleet with a lower production, brown shrimp is amongst the top 10 species (in terms of supply as well as of value) for the Flemish fleet (Anonymous 2009b). In 2010, Flanders' fishermen caught 1563 tonnes of shrimp, whereas 588 tonnes were landed in Flanders' harbours. This could count for approximately 29 million euro. Although 2010 was a relatively good year, shrimp fishermen earned a lower amount of money per fishing day in comparison with fishermen targeting other fish species. The shrimp landed in Belgian harbours by Flemish fishermen are artisanally processed on board of the vessels.

Microbial quality and spoilage of crustaceans

Crustaceans such as brown shrimp are prone to rapid microbiological spoilage. This is due to the neutral pH, high water activity (a_w), and a high NPN fraction (Liston 1980; Mendes et al. 2002a). However, to date not much research on the microbiological quality or spoilage of brown shrimp has been carried out. Since cooked shrimp are ready-to-eat products, it is important to follow the microbiological quality and the presence of pathogens. In a study performed in The Netherlands in 1991, it was seen that many samples of especially brown shrimp exceeded the maximal microbiological contamination degree based on aerobic counts (at 30°C) of 10^6 (CODEX norm) and even 10^7 log cfu/g (ICMSF norm) (Jonker et al. 1992). This is in contrast to tropical shrimp, where much lower concentrations of preservatives may be used. During the years, the microbiological contamination of shrimp has improved. In 1981, 89% of the tested shrimp samples would be unacceptable for purchase, wheras in 1992 this number was already reduced to 27% and in 2000 only 13% was considered unacceptable (Jonker et al. 2000). The microbiological levels and norms concerning the conservation of unpeeled and peeled brown shrimp are listed in Table 2.6 and 2.7.

Table 2. 7. Microbiological levels and norms concerning shrimp (Bon 1996).

Mesophilic aerobic total count (cfu/g)	Description
10^2	Shrimp, immediately after cooking
10^3	Rinsing and cooling of the shrimp with clean seawater
10^4	m= below limit maximal count of unpeeled shrimp
$5x\ 10^4$	m= below limit maximal count of peeled shrimp
10^5	M= upper limit maximal count of unpeeled shrimp
$5x\ 10^5$	M= upper limit maximal count of peeled shrimp
10^6	Sensorial limit for rejection of shrimp without preservatives
10^7	Sensorial limit for rejection of shrimp with preservatives

Influence of handling and processing of fish on the microbiota

Processing on board

On board processing starts with immediate sorting of the shrimp and the fish caught in the nets. During sorting, fish is manually or mechanically removed from the shrimp fraction. The shrimp are placed in baskets of about 20 kilo. One by one, the baskets are poured in a large cooking jar on deck in approximately 100 litres of boiling seawater with an extra cup of sea salt added. When the 20 kilos of cold shrimp are poured into the boiling water, the temperature immediately drops to 86 - 92°C. The shrimp are 'cooked' for a maximum of 2 to 4 minutes. In a study observing the cooking process of white shrimp (*Penaeus indicus*), it was found that a boiling time of 3 minutes is enough to reduce the number of microorganisms with 4 log and to inactivate enzymes causing melanosis in boiled shrimp (Niamnuy et al. 2007). For brown shrimp, the time of cooking is considered as a delicate balance between microbiological decontamination (degree of maximal contamination 10^5 $\log_{10}$ cfu/g) and shrimp quality. Texture is considered to be the most important sensorial quality attribute since it may change dramatically during extended cooking, while the characteristic shrimp flavour develops relatively early during the process and does not change substantially after prolonged heating (Ma et al. 1983; Niamnuy et al. 2007). After cooking, the shrimp are immediately cooled down for about 5 minutes in a rinsing machine where cold clean seawater is poured over the cooked shrimp. In theory, clean seawater means seawater, salt or brackish water that does not cause microbiological recontamination. However in practice, cooling with seawater recontaminates the shrimp with psychrophilic microbiota. Van Spreekens and de Man, however, reported that this recontamination also occurs when the shrimp are cooled to the air or by ice (1970). This cooling decreases the shrimp's temperature to approximately 15°C. The cooled shrimp are stored below deck in a cold room covered with ice until landing the following morning. During storage on board as well as on land, temperature is very important in relation to shelf life; the lower the temperature, the longer the shelf life (Dalgaard and Jorgensen 2000). Different ways of icing and rapid cooling are reflected in the shelf life; Zeng et al. (2005), showed that storage in liquid ice and superchilling (-1.5°C) are beneficial for the shrimp's shelf life.

Processing on land

Once on land, the shrimp are landed near the fish auction where a quality control is performed based on temperature and microbiological contamination. The freshness and size category of

the shrimp is determined based on a few criteria (Anonymous 1996) as described in Tables 2.8 and 2.9.

Between the cooking of shrimp and consumption, diverse processing steps occur which all may contribute to post-catch contamination of the product. The longer the processing line/time, the higher the contamination degree (Wiedemeijer and Pateer 1984). Once the shrimp enter the fish auction, they are generally immediately treated with preservatives in order to prolong the shelf life. Mostly benzoic acid (E210) and sorbic acid (E200) or the derived salts (sodium benzoate E211 and sodium sorbate E201) are added. Since those products can have a negative consequence for human health, the maximum concentration allowed is regulated by law. For most shrimp species, 2 grams per kilo is the upper limit; for *Crangon crangon* shrimp the legal limit is three times higher (0.6%) in accordance with the EU standard (Anonymous 1995). Often organic acids such as citric acid (E330), acetic acid

Table 2. 8. Freshness parameters of common shrimp (*Crangon crangon*) as described by the Council Regulations No 2406/96 (Anonymous 1996).

	Criteria **Freshness category**	
	Extra	**A**
Minimum requirements	▪ Surface of shell: moist and shiny Shrimp must fall out separately when transferred from one container to another ▪ Flesh must be free from any foreign odour ▪ Shrimp must be free from sand, mucus and other foreign bodies	▪ The same as for Extra category
Appearance of shrimp with shell	▪ Clear reddish-pink in colour with small white flecks; pectoral part of shell predominantly light in colour	▪ Ranging in colour from slightly washed-out reddish-pink to bluish-red with white flecks; pectoral part of shell should be light coloured tending towards grey
Condition of flesh during and after peeling	▪ Shells easily with only technically unavoidable losses of flesh ▪ Firm but not tough	▪ Shells less easily with small losses of flesh ▪ Less firm, slightly tough
Fragments	▪ Occasionally fragments of shrimp allowed	▪ Small quantity of fragments of shrimp allowed
Smell	▪ Fresh seaweed, slightly sweet smell	▪ Acidulous; no smell of seaweed

Table 2. 9. Quality parameters concerning the size of common shrimp (*Crangon crangon*) as described by the Council Regulations No 2406/96 (Anonymous 1996).

Scale of size for Crangonid shrimp (*Crangon crangon*)	
Size	**Kg/fish** [1]
1	6,8 mm and over [2]
2	6,5 mm and over

[1] The categories of weights include fish from the lower limit up to, but excluding, the upper limit; [2] Width of shell

(E260), lactic acid (E270), glucono delta-lactone (E575) or the derived salts thereof are added too since the above-listed preservatives function more effective at lower pH values. To avoid discolouration, sodium metabisulfite (E223) is often added. Another possibility to decrease the microbiological contamination is irradiation. Irradiation of frozen shrimp is limited to a few countries such as France, The Netherlands, and Belgium (Diehl 1992). Adding preservatives in high concentrations is mainly needed because of the extra microbiological contamination pressure during the peeling process. Brown shrimp are currently still hand-peeled in low-cost countries, such as Morocco, in large temperature-regulated peeling centres. For peeling in low cost countries, lorries drive up and down with shrimp kept in climate-controlled conditions. Approximately two weeks later, the hand-peeled shrimp return and can be packed in modified atmosphere packages (MAP). A MAP package with 45% CO_2, 5% O_2 and 50% N_2 seems to be the most effective to preserve pink shrimp quality up to 9 days compared to 4 to 7 days for ice storage (with pre-treatment with preservatives) (Goncalves et al. 2003).

Some of the shrimp are processed according to the PURUS standards, indicating that no preservatives are added (http://hirammedia.typepad.com/purus/purus.html (16/01/2012)). PURUS indicates artisanal products that have received a regional product label. Those shrimp are offered on the market within 24 hours after catch. The shrimp are size-sorted and are offered unpeeled and peeled in MAP package. Peeling of the shrimp generally decreases the total count by 0.5 log, indicating that the carapax of the shrimp is the most contaminated area. However, cross-contamination between the carapax and the inner flesh of the shrimp during peeling seems unavoidable. The peeled shrimp are locally machine peeled.

Enterobacteriaceae may indicate unhygienic processing circumstances (Jonker et al. 2000).

New trends in shrimp fishery

To date, experiments with electrical fishing gear are going on (Polet et al. 2005), because the traditional beam trawling fishing technique causes stress on the seabed and all its inhabitants and results in a very large by-catch.

Nowadays, some shrimp are kept alive on board until sold (http://www.northsealife.be/ (22/08/2011). Since further processing occurs on land, microbiological contamination and all processing steps can be better monitored

Population shifts during different storage conditions

To supply all year around, shrimp are often freeze-stored. Processing procedures such as freezing/thawing and long frozen storage influence the colour and texture of brown shrimp. Freezing/thawing has a stronger influence on texture (hardness, chewiness, resilience, springiness and cohesiveness) and colour stronger than prolonged frozen storage (Schubring 2002). According to Makarios-Laham and Lee (1993), psychrophilic microorganisms may contribute to deterioration of seafood even during frozen storage. In the latter study, slow microbiological growth was measurable at -5°C and -8°C. Growth near subfreezing temperatures was mainly by Gram-negative, oxidase- and catalase-positive, coccobacilli-shaped cells; which were identified as *Psychrobacter phenylpyruvicus* by API tests (Tsironi et al. 2009).

SSOs of crustaceans

On brown shrimp, no records of SSO identification are reported. However, microorganisms such as *Pseudomonas* sp., *Shewanella* sp., *Photobacterium phosphoreum* and *Brochothrix thermosphacta* are the common SSOs found on cold-stored crustaceans (Dalgaard 1995b; Gram and Huss 1996; Mejlholm et al. 2005; Vogel et al. 2005). However, the microbiota of chilled, lightly preserved (e.g. brined) shrimp such as *Pandalus borealis* seem often to be dominated by LAB (Dalgaard and Jorgensen 2000; Einarsson and Lauzon 1995; Jeppesen and Huss 1993). Especially *Carnobacterium divergens*, *C. maltaromaticum* and other unidentified *Carnobacterium*-like bacteria were isolated from spoilage associations of chilled seafood (Dalgaard et al. 2003; Mauguin and Novel 1994). LAB can originate from the intestinal content of live fish and shellfish (Ringo and Gatesoupe 1998).

Next to the presence of some typical species, such as Pseudomonads and *Aeromonas* spp., the presence of pathogens is important for the microbiological quality research of shrimp. Pathogens can be present from initial contamination but are mostly eliminated by cooking.

Nevertheless, post-catch contamination may occur due to the cooling water or during peeling or packaging due to unhygienic conditions. The legal limits for pathogens such as *E. coli* are listed in Table 2.6. Also inappropriate storage temperature can threaten human health. For instance, *Vibrio* spp. are present in seawater, and can potentially recontaminate the shrimp via cooling water (Jonker et al., 2000).

Biochemical changes in crustaceans and quality

Endogenous enzymes and microbiological activities have a special role in the deterioration of crustaceans (Finne 1982). The determination of several chemical compounds such as TVBs, as well as nucleotides and their breakdown products, have been suggested as criterium for the evaluation of crustacean quality (Cobb and Mathews 1973; Mendes et al. 2002b).

2.3.2. Ray (*Raja* sp.)

Biology, fishery and the importance of ray in Belgium

In several European countries, including Belgium, ray (*Raja* sp.) represents the most commercialised elasmobranch fishery product. Ray is mainly caught in sandy European coastal shelf areas (Anonymous 2006) and is often a by-catch product of the fishery of sole and other commercial important flatfish. In the North Sea, especially thornback ray (*R. clavata*), spotted ray (*R. montagui*), and blonde ray (*R. brachyura*) are caught. In Belgium, ray belongs to the top three of fishery products in terms of supply with an annual catch around 1500 tonnes the last years. In terms of value, ray belongs to the top 10 (Anonymous 2009a).

(Microbial) quality and spoilage

A typical fish spoilage phenomenon prior to *rigor mortis* is the production of slime in certain skin cells of the fish. The secreted quantity of slime depends on the fish species, with fish that have poorly developed scales secreting more. The secretion process stops with the onset of *rigor mortis*. This slime contains a high amount of nitrogenous compounds, which provides nutrients for the initial microbiota (see 2.1.3.). This slime will spoil very quickly and will facilitate the deeper microbiological penetration into the fish flesh.

The initial microbiological contamination of fish varies between 10^2 to 10^7 cfu/cm² on the skin in the outer slime layer and between 10^3 and 10^9 cfu/g on the gills and in the intestines, while the fish flesh is considered to be sterile (Shewan 1962).

Influence of handling and processing of (elasmobranch) fish on the microbiota

The early handling and processing of fish comprises next to icing (see 2.1.3.) also the removal of intestines (gutting) of freshly caught fish. Gutting has several benefits, including a decrease in (mesophilic) aerobic counts, and a lower amount of H_2S-producing bacteria and *Enterobacteriaceae* (Erkan and Ozden 2008; Papadopoulos et al. 2003). Some studies, however, did not find any significant decrease in microbiological numbers, but did find that the SSOs were detected faster and in higher amounts in gutted fish (Erkan and Ozden 2008;Karl and Meyer 2007). This observation might possibly be explained by cross-contamination during gutting.

Also, washing of the fish with seawater reduced the total viable counts and can be correlated with the removal of the outer slime layer, a source of nutrients for microorganisms (Erkan 2007). The skin of fish also has a protective function; for instance filleted fish will have a faster increase of the microbiological counts than whole fish due to the removal of the skin which may slow down the microbiological penetration (Poli et al. 2006).

A characteristic of all elasmobranch fish is the occurrence of a high level (1.0 to 2.5%) urea in the muscles, blood, organs and skeleton (Huss 1995; Vyncke 1978a). During spoilage, urea breaks down with the formation of ammonia (see further). Often, the capture of ray in distant fishing areas means that the time elapsed between catch and arrival at the fish auction may vary from one to eight days. Therefore, intense and optimal refrigeration is a must to maintain fish of the highest quality (Bilinski et al. 1983; Múgica et al. 2007; Ocano-Higuera et al. 2011; Ravesi et al. 1985; Vyncke 1978a). Moreover, the formation of ammonia may limit the commercialisation period of elasmobranch fish (Finne 1992; Vyncke 1978a). Hence, Vyncke set the borderline of acceptability of ammonia at 60-70 mg per 100 g ray (1978a). This fast deterioration caused that 1.5 to 4.0% of the rays docked in Belgian harbours were no longer suitable for consumption (Anonymous 2009a). Immediate icing of elasmobranch fish is essential to obtain a high quality product for a reasonable period of shelf life. Several studies performed on elasmobranch fish have shown that correct icing delays the formation of ammonia and TMA in the fish muscle; even a small change of 1.5°C (flake ice or slurry ice) will have an effect on the shelf life (Bilinski et al. 1983; Múgica et al. 2007; Ocano-Higuera et al. 2011; Ravesi et al. 1985; Vyncke 1978a). Once the rays are landed, a quality control based on sensorial characteristics is performed to determine whether the fish quality is high enough. This quality control and measurement of the size order is based on the parameters described in Tables 2. 10. and 2.11. according to the EU scheme (Anonymous 1996).

Table 2. 10. Freshness parameters of ray species (*Raja* sp.) as described by the Council Regulations No 2406/96 (Anonymous 1996).

	Criteria			
	Freshness category			Not admitted
	Extra	A	B	
Eye	Convex, very bright and iridescent; small pupils	Convex and slightly sunken; loss of brightness and iridescence, oval pupils	Flat, dull	Concave yellowish
Appearance	In *rigor mortis* or partially in rigor; small quantity of clear mucus present on skin	Beyond rigor stage; no mucus on skin and especially in mouth and gill openings	Some mucus in mouth and on gill openings; slightly flattened jaw	Large quantities of mucus in mouth and on gill openings
Smell	Seaweed smell	No smell or very slight stale but not an ammonia smell	Slight ammonia smell; sour	Pungent ammonia smell
Specific or additional criteria for skate				
Skin	Bright, iridescent and shiny pigmentation, aqueous mucus	Bright pigmentation, aqueous mucus	Pigmentation in the process of becoming discoloured and dull, opaque mucus	Discolouration, skin creased, thick mucus
Texture of the flesh	Firm and elastic	Firm	Soft	Flaccid
Aspect	Edge of the fins translucent and curved	Stiff fins	Soft	Drooping
Belly	White and shiny with mauvish edge around the fins	White and shiny with red patches only around the fins	White and dull, with numerous red or yellow patches	Yellow to greenish bellies red patches in the flesh itself

Table 2. 11. Quality parameters concerning size of ray species (*Raja* sp.) as described by the Council Regulations No 2406/96 (Anonymous 1996).

	Scale of weights	
Species	**Size**	**Kg/fish**
Whole skates	1	5 and over
	2	3 to 5
	3	1 to 3
	4	0.3 to 1
Skate wings	1	3 and over
	2	0.5 to 3

Since ray is offered as a fresh fishery product in Belgium, not much further processing is involved. The general processing concerns the gutting of the fish which is performed shortly after catch and removal of the wings. Proper gutting prolongs the shelf life, while an inappropriate gutting accelerates microbiological contamination and growth (Ravesi et al. 1985). The ray wings are sold to consumers and are mainly stored on ice until purchased; in some supermarkets they are MAP packed. In some European countries, such as Spain, ray meat is also frozen and can be used in canned fish products (Pastoriza and Sampedro 1993).

SSOs of elasmobranch fish

While 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 its possible effect on the degradation of urea to ammonia.

Biochemical changes of elasmobranch fish under aerobic conditions

Biochemical composition of elasmobranch fish

The NPN fraction in the fish muscle causes the rapid decay by microbiological activities of fish. Especially elasmobranchs (such as rays) contain about twice as high a concentration of soluble components (e.g. urea and TMAO (see 2.1.4. and Fig. 2.3) as do other fish (Huss 1995). In elasmobranch fish such as sharks and ray, a high concentration of urea is stored in the cartilaginous skeleton of the fish. This urea can be converted to ammonia due to autolytic and/or microbiological activity (Fig 2.7). Ammonia can also be formed together with H_2S as a result of the conversion of cysteine to pyruvate by the enzyme cysteine desulfhydrase (Gill 1982). The release of large amounts of ammonia will contribute to the development of spoilage odours. The main cause of ammonia production from urea (autolytic or microbiological) is currently not yet known (Múgica et al. 2007).

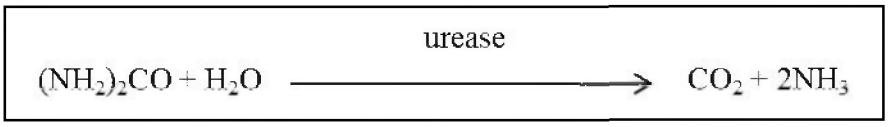

Fig. 2. 7. Conversion of urea to ammonia due to urease activity.

Due to the high concentration of urea (2000 mg) in elasmobranch fish (Huss 1995; Vyncke 1978a), the main reason for rejection is the production of ammonia. In fish other than elasmobranch fish, the post-mortem ammonia production arises from the enzymatic deamination of proteins, ammonia acids, and other nitrogen compounds. In elasmobranchs, the ammonia is mainly formed by the enzymatic degradation of urea. The enzyme responsible for this activity is thought to be urease, present in certain bacteria (Vyncke 1978a). Research concerning the quality of elasmobranch fish has shown that the fraction of urease-positive bacteria comprises 3 to 20 percent of the total aerobic count on elasmobranch fish stored on ice (Ravesi et al. 1985). When considered spoiled, the fraction of these urease-positive bacteria was 6.3 log cfu/g, while the total microbiological count at the point of spoilage was around 8 log cfu/g (Moyer et al. 1959; Ravesi et al. 1985). When dipping ray fillets in citric acid, a product known for its preservative action, the shelf life increased with a few days (Vyncke 1978b), indicating the possible microbiological role in spoilage. However, a study

by Mugica et al. (2007) found that the ammonia production was correlated more with the activity of the endogenous mechanisms involved in the degradation of proteins and non-protein-nitrogen compounds (the NPN fraction), rather than with the activity of microorganisms. This was concluded due to the very low microbiological count when the fish was rejected by sensorial criteria (Múgica et al. 2007). Therefore, the main cause of ammonia production (autolytic or microbiological) is not exactly known.

The ammonia production in elasmobranch fish causes an increase in pH beyond the optimum for TMAO reductase (pH 7.2-7.4) (Castell and Snow 1949). Therefore, the production of ammonia could explain the rather low production of TMA during spoilage although the precursor TMAO is present in high levels (Elliot 1952). The concentration of TMAO in elasmobranch fish is two to three times higher than in cod (500-1000mg in elasmobranch fish compared to 350mg in cod), whereas in cod TMA production is the main cause of the spoilage (putrid fishy smell) in contrast to elasmobranch fish (Huss 1995).

PART I

SEAFOOD QUALITY ASSESSMENTS: EVALUATION OF TRADITIONAL DETECTION TECHNIQUES

Preamble

Correct and profound seafood quality research depends on the detection of the microbiota present during storage and spoilage. Independent of the purpose (purchase agreements, governmental control, research for shelf life improvement by SSO identification, ...) the first step is always creating a profound overview of the microbiota. In order to detect and identify the dominant microbiota on brown shrimp and ray, we first need to evaluate the general growth media currently used in seafood quality research.

CHAPTER 3

SEAFOOD QUALITY ANALYSIS: MOLECULAR IDENTIFICATION OF DOMINANT MICROBIOTA AFTER ICE STORAGE ON SEVERAL GENERAL GROWTH MEDIA

Redrafted from:

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Chapter 3. Seafood quality analysis: molecular identification of dominant microbiota after ice storage on several general growth media

Abstract

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

I. Introduction

Fresh seafood is very sensitive to spoilage. The main factors limiting shelf life are enzyme and microbiological activities. Freshly caught fish are naturally contaminated with a diversity of microbiota which depends among other things on the environment, water temperature, area of catch, handling and processing procedures (Jay, 1986). During storage certain genera are able to grow very well and population shifts may occur depending on the storage conditions. As described in chapter 2, quality control and potential shelf life of fish is currently still often estimated based on the total aerobic psychotolerant count (APC). Standards, guidelines and specifications as part of purchase agreements of chilled fish quality accept an APC of 10^6 cfu/g for human consumption (Anonymous, 1986). When reaching an APC of 10^7-10^8 cfu/g, spoilage generally becomes organoleptically detectable (Liston, 1980). The ISO reference medium for the psychotolerant enumeration of microorganisms on food (ISO 17410; International Organization for Standardization, 2001), including fish, is plate count agar (PCA) without addition of extra salt or minerals. However, studies have reported that an APC

underestimation of 1 $\log_{10}$ cfu/g on PCA is plausible in comparison with growth media containing sodium chloride (NaCl) such as Long and Hammer agar (LH; Joffraud & Leroi, 2000; Van Spreekens, 1974), which is recommended by the NMKL 184 (Nordic Committee of Food Analysis, 2006) method for the enumeration of psychotolerant microorganisms in seafood.

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

II. Materials and methods

2.1. Raw material, preparation and storage

Eleven fresh, gutted fish samples were taken from a supermarket's supply centre. Following fish species were selected: plaice (*Pleuronectes platessa*), common sole (*Solea solea*), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), salmon (*Salmo salar*), whiting (*Merlangius merlangus*), mackerel (*Scomber scombrus*), pangasius (*Pangasius pangasius*), ray (*Raja* sp.), cod (*Gadus morhua*) and angler fish (*Lophius piscatorius*). Small fish were collected whole, a 200-gram piece was taken from the bigger fish (cod, salmon and angler fish). Whiting and pangasius were only available as fillets. Ray, sole and angler fish had their skin removed at purchase. The samples were put in sterile stomacher bags and transported on ice to the lab. Upon arrival at the lab (T_1), a piece of 2 by 2 cm (equalling 10 g) was aseptically excised for microbiological analysis, and the remaining fish was stored in a sterile bag on ice at 0 ± 0.5 °C. A second sample was taken at T_2, namely after 7 days or 4 days (ray) of ice storage.

2.2. Cultivation and isolation of microorganisms from the fish samples

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

2.3. Replication of the cultivated microorganisms on different growth media

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

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

2.4.1. DNA extraction

After replication, a selection of 121 isolates with different colony morphology at T_2 were selected based on their inability to grow on one of the media. These strains were purified and DNA extraction was performed using a modified Flamm method (Flamm et al., 1984), adjusted with lysostaphine (0.5 mg/µl) and mutanolysine-lysozyme solution (1 U/µl mutanolysine, 2.5 mg/ml lysozyme) which were dissolved in HPLC water and TE-buffer (0.05 M Tris, 0.02 M EDTA, pH 8), respectively, and were added to the pellet of pure culture at the start of the DNA extraction.

2.4.2. Rep-PCR

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

2.4.3. Identification of the microbial isolates by sequence analysis

A 1500 bp fragment of the 16S rRNA gene was amplified by PCR using the conserved primers 16F27 and 16R1522 (Brosius et al., 1978). Amplification was performed as follows: 30 cycles at 94 °C for 15 s, 57 °C for 15 s and 72 °C for 30 s followed by an elongation step at 72 °C for 8 min. Further identification was performed by *gyrB* gene sequence analysis. A

1500 bp fragment of the *gyrB* gene was amplified by PCR using the universal primers UP1 and UP2r following the protocol of Yamamoto and Harayama (1995). All PCR products were purified for sequencing with a High Pure PCR product purification kit (Roche) according to manufacturer's protocol and stored at -20 °C. The quality and quantity of the purified PCR products were verified on a 1.5% agarose gel.

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

2.5. Differences in the microbiological community by PCR-DGGE analysis

A complete plate swab was performed using an inoculation loop. The dilution used for the plate swab depended on the APC of the fish sample. The pellets were washed twice with 1x PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4 and 6.4 mM Na_2HPO_2 (pH 7.4)) and

stored at -20 °C. A DNA extraction was performed using the Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. This DNA, diluted in 200 µl elution buffer (QIAGEN) was used for further analysis by denaturant gradient gel electrophoresis (PCR-DGGE).

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

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

III. Results

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

Averages for duplicate plate counts on PCA are presented as $\log_{10}$ cfu/g (Table 3.1). At T_1, the APC of the fish samples ranged from 2.7 to 6.1 $\log_{10}$ cfu/g. At this point, APC was highest for the ray sample and lowest for salmon. At T_2, APC increased and ranged from 4.4 to 7.2 $\log_{10}$ cfu/g. The sample of pangasius showed an increase of less than 0.5 $\log_{10}$, while those of

angler fish and mackerel increased by more than 2 log_{10}. The APC of the other samples increased between 1 and 2 log_{10}.

Table 3.1. The total aerobic psychotolerant count (APC) (log_{10} cfu/g) on Plate Count Agar (PCA) (mean of duplicate platings) for all 11 fish samples at the time of arrival (T_1) and after 7 days or 4 days (ray) of ice storage (T_2).

	Total aerobic psychotolerant count (APC) (log_{10} cfu/g)										
Sampling	**Salmon**	**Whiting**	**Ray**	**Pangasius**	**Cod**	**Plaice**	**Sole**	**Sea bass**	**Angler fish**	**Mackerel**	**Sea bream**
T_1	2.7	5.5	6.1	4.9	3.6	4.0	5.3	4.5	4.9	4.3	5.8
T_2	4.4	6.8	6.6	5.2	4.8	6.0	6.9	6.4	7.2	6.8	6.8

When comparing PCA, MA, LH and IA at T_1 (Fig. 3.1) and after ice storage (T_2) (Fig. 3.2), PCA generally showed a lower APC than the salt-containing media (MA, LH and IA), but some counts were equal. Although APC between several batches of the same fish species also showed some differences, the difference between APC on PCA and the other media was always more pronounced. Student t-tests showed a statistically significant difference, except between LH and MA at T_1 and T_2 and between PCA and IA at T_1.

The highest difference in APC at T_1 was observed between PCA and LH, with those on PCA being 0.3 to 1.1 log_{10} cfu/g lower than on LH (Fig. 3.1). Differences in APC lower than 0.5 log_{10} were interpreted as within standard plate counting error and are not shown. Five samples showed a 0.5 to 1 log_{10} lower APC on PCA than on LH, and one sample exceeded a 1 log_{10} difference. Differences between MA, LH and IA were lower than 0.5 log_{10} except for two samples (ray and plaice) where IA had a lower APC compared to MA and LH.

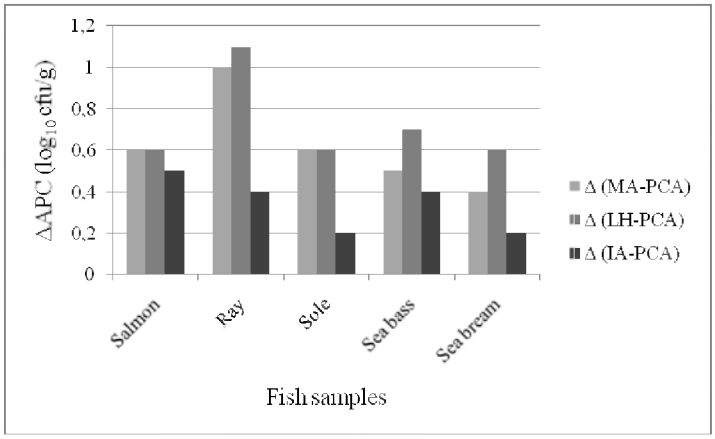

Fig. 3.1. Differences (Δ) in total aerobic psychotolerant count (APC) (log_{10} cfu/g) between MA, LH, IA and PCA growth media at arrival (T_1) of fish samples. Only those fish samples are shown where a higher difference than 0.5 log_{10} cfu/g between PCA and the salt-containing media (MA, LH and IA) was observed.

At T_2, the difference in APC between PCA and the other media (MA, LH and IA) increased for most fish samples. APC on LH and MA was almost equal, with both being 0.5 to 1.8 $\log_{10}$ higher than on PCA (Fig. 3.2). Compared to T_1, more samples showed a significant difference between PCA and LH/MA, and only two samples (angler fish and mackerel) had an APC difference lower than 0.5 $\log_{10}$. Five samples (whiting, pangasius, plaice, sea bass and sea bream) showed a 0.5 to 1 $\log_{10}$ difference between PCA and LH/MA, while four samples (salmon, ray, cod and sole) showed a difference more than 1 $\log_{10}$. Five samples showed an APC difference of 0.5 $\log_{10}$ or more between LH and IA (salmon, whiting, ray, cod and sole).

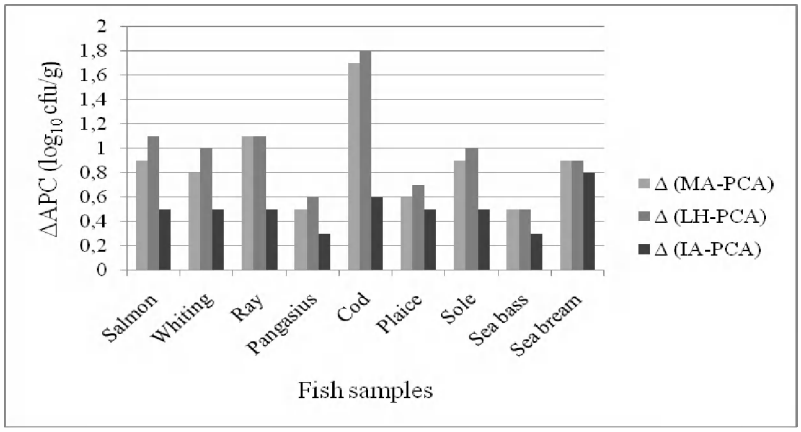

Fig. 3.2. Differences (Δ) in total aerobic psychotolerant count (APC) ($\log_{10}$ cfu/g) between MA, LH, IA and PCA growth media at T_2 (i.e., after 7 days or 4 days (ray) of ice storage) of fish samples. Only those fish samples are shown where a higher difference than 0.5 $\log_{10}$ cfu/g between PCA and the salt-containing media (MA, LH and IA) was observed.

3.2. Replication of the cultivated microorganisms on different growth media

Replication showed a high percentage of colonies unable to grow on at least one medium (Table 3.2). On MA, LH and IA, most colonies were able to grow.

At T_1, all fish samples, excluding the pangasius sample, contained microorganisms unable to grow on all tested media. Of those samples originally incubated on MA or LH, 10 to 92% of the colonies, depending on the fish sample, could not grow on PCA after replication. Thirteen to 68% of the colonies from samples originally incubated on IA were unable to grow on PCA. One to 53% of the colonies from samples originally incubated on PCA could not grow on one or more of the salt-containing media. At T_2, shifts were noticeable. The number of non-growing colonies replicated to PCA increased for seven fish samples (salmon, whiting, ray, pangasius, cod, sole and sea bream). As for the other four fish samples, the number of non-growing replicated colonies was equal or lower than at T_1. On the other hand, the percentage

of colonies taken from PCA that did not grow on the salt-containing media was mostly much lower at T_2 than at T_1.

Table 3.2. Percentages of colonies non-growing on the different media (PCA, MA, LH and IA) after replication at both time points, T_1 (sampling at the time of arrival) and T_2 (after 7 or 4 days (ray) of ice storage). X/Y: The growth medium Y from which the colonies are replicated onto growth medium X. Between brackets are the number of colonies that have been transferred by replication as mentioned. When replicated from PCA onto LH, MA or IA, the number and media indicated after the asterisk refer to the percentage of colonies not growing on the mentioned salt-containing medium. The average percentage of non-growing colonies from the total pool of fish species is given in the last row.

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

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

After replication, a selection of 121 isolates with different colony morphology were selected based on their inability to grow on at least one of the tested media. The colonies were collected from the plates at T_2, since mainly those microorganisms present during spoilage were of interest. These isolates clustered based on their $(GTG)_5$ fingerprint (Supplementary Fig. 3.1), showed a large variety, with some large clusters present. These clusters were

visually defined. PCR-reproducibility ranged from 95.3 to 99.0% similarity (Pearson correlation).

From this cluster analysis, 39 representatives were selected, with a minimum of 2 isolates per cluster, and identified based on their partial 16S rRNA and *gyrB* gene sequence. Twenty-one different species were identified (Table 3.3). Using 16S rRNA gene analysis, the representative isolates could mostly be allocated to species complexes after BLAST search with the EzTaxon database. Further identification to species level was obtained by *gyrB* sequence analysis. However, not all *gyrB* sequences of all species were available. In these cases, 16S rRNA gene based identification on the genus and/or species complex level was used to select type strains of species not present in any accessible database of *gyr*B sequences. Phylogenetic trees were constructed with these extra type strains included together with the known *gyr*B sequences and the representative isolates.

Members of the genera *Photobacterium*, *Shewanella*, *Vibrio*/*Aliivibrio*, *Pseudoalteromonas*, *Psychrobacter* and *Pseudomonas* were unable to grow on PCA, but tended to grow very well on fish during ice storage as evidenced from their isolation on the other growth media at T_2 (Table 3.3). In particular, *Shewanella frigidimarina* and *Pseudoalteromonas nigrifaciens* were abundantly present in this study, with 47 and 33 isolates found, respectively. On the other hand, some microorganisms could only grow on PCA, i.e. some species of the genera *Psychrobacter*, *Pseudomonas* and *Acinetobacter*. Within the genus *Psychrobacter*, *Psychrobacter fozii* and *Psychrobacter maritimus* could not grow on LH, and *Psychrobacter cibarius* could not grow on either of the salt-containing media. *Psychrobacter proteolyticus* and an isolate identified as *Psychrobacter cibarius* by *gyr*B sequence analysis (*Psychrobacter cibarius*-like species) were, however, unable to grow on PCA. Within the genus *Pseudomonas* two species were identified. *Pseudomonas fragi* was unable to grow on salt-containing media, while *Pseudomonas fluorescens* was unable to grow on PCA. Some species of the genus *Flavobacterium* and *Janthinobacterium* were unable to grow on MA. *Brochothrix thermospacta* was unable to grow on IA.

Table 3.3. Identification of 121 isolates with different colony morphology from the 11 fish samples after 7 or 4 days (ray) of ice storage, which did not grow on a specific growth medium. The (range of) similarity values (*gyr*B) against the type strains of the species (exceptions are mentioned in footnotes) are listed in the second column, when the *gyr*B sequence could not be used, the 16S rRNA similarity values are used. The abundance of isolation of these species is listed in the third column by the number of isolates. The fish samples on which the microorganisms are found are in the third column.

Identification	Similarity values (%)	# isolates	From fish sample	Not growing on
Photobacterium				
P. phosphoreum	99.6	4	salmon, cod, whiting	PCA
P. iliopiscicarium	99.1	3	sole, plaice, whiting	PCA
Shewanella				
S. frigidimarina	94.6 - 99.2[1]	47	all fish	PCA
S. vesiculosa	98.5 - 99.5	5	angler fish, plaice, sole, salmon, pangasius	PCA
S. baltica	98.7 – 99.1	3	angler fish, cod, mackerel	PCA
S. glacialipiscicola	98.7	1	plaice	PCA
Vibrio/Aliivibrio				
V. litoralis	97.0 (16S)	2	mackerel, sole	PCA
A. logei	96.2	1	salmon	PCA
Pseudoalteromonas				
Psa. nigrifaciens	99.8	33	all fish except cod	PCA
Psychrobacter				
Psb. cibarius-like[2]	/	1	mackerel	PCA
Psb. proteolyticus	94.0 - 95.2	2	angler fish	PCA
Psb. fozii	/[3]	1	angler fish	LH
Psb. maritimus	/[4]	1	angler fish	LH
Psb. cibarius	/[5]	1	mackerel	MA, LH & IA
Pseudomonas				
Ps. fragi	92.2 – 93.3	5	angler fish, mackerel, sea bass	MA, LH & IA
Ps. fluorescens	89.2	1	gilthead sea bream	PCA
Acinetobacter				
Acinetobacter sp.	80.8[6]	1	mackerel	MA, LH & IA
Flavobacterium				
F. hydatis	97.7 – 98.3	4	salmon, plaice, cod	MA
F. hercynium	96.6 (16S)	2	angler fish, gilthead sea bream	MA
Janthinobacterium				
J. lividum	95.6	1	cod	MA
Brochothrix				
B. thermosphacta	99.6 (16S)	2	gilthead sea bream, sole	IA

[1]The similarity values could not be calculated based on a type strain, but were calculated based on an environment isolate (NCIMB 400). Phylogenetic tree analysis confirmed the identification.
[2] The isolate was identified based on *gyr*B gene sequence and phylogenetic tree analysis. FASTA results gave 96.2% similarity with the *Psb. immobilis* type strain, however, phylogenetic tree analysis with inclusion of extra type strains confirmed a higher similarity with *Psb. cibarius*. Sequence comparison between both isolates and the type strain showed several mismatches with the sequence of the *Psb. cibarius*-like isolate.
[3] The isolate showed 93.6% similarity with *Psb. luti* type strain, but phylogenetic tree analysis gave a higher similarity with *Psb. fozii*.
[4] FASTA results gave a similarity value of 93.6% with the *Psb. luti* type strain, phylogenetic tree analysis gave a higher similarity with the *Psb. maritimus* type strain.
[5] FASTA results gave a similarity value of 97.4% with the *Psb. immobilis* type strain, phylogenetic tree analysis gave a higher similarity with the *Psb. cibarius* type strain.
[6] The *Acinetobacter* isolate showed 80.8% similarity with *the Acetinobacter johnsonii* type strain.

3.4. Differences in the microbiological community on the different growth media by PCR-DGGE analysis

The PCR-DGGE analysis of the plate swabs of mackerel from the different growth media at T_1 and T_2 is shown in Fig. 3.3A. A clear difference in band patterns was observed between PCA and the other media, indicating a different microbiota on PCA at both time points. In comparison to MA, LH and IA, PCA exhibited only a very small number of bands especially at T_1. At T_2, the number of bands for PCA highly increased but the pattern observed from PCA was still quite different from the other media. The PCR-DGGE pattern from the salt-containing media showed some variation between both time points; nevertheless, the difference was rather small compared to the difference on PCA. A similar clearly distinct pattern of PCA from the other media was also observed for several other fish samples such as plaice, salmon, gilthead sea bream, whiting, sea bass, sole and cod. However, not all fish samples showed such a clear difference in PCR-DGGE pattern between PCA and the other media. PCR-DGGE analysis of the brackish water fish pangasius (Fig. 3.3B) showed that besides a few bands, the pattern obtained from PCA plate swabs was more similar to the salt-containing media at T_1, indicating that the dominant microbiota on all media was more or less the same. At T_2, a shift in PCR-DGGE pattern was noticed where the differences between PCA and the salt-containing media became much larger. Rather subtle differences in dominant microbiota were also observed for the fish samples of angler fish and ray.

IV. Discussion

4.1. Quantitative differences between the media

Most samples of fish species examined, except three (salmon, pangasius and cod), reached an aerobic psychotolerant count of 6 $\log_{10}$ cfu/g on PCA at T_2. At the same time, an APC of 7 $\log_{10}$ cfu/g was counted on the salt-containing media indicating that these fish are definitely no longer suitable for purchase. On the other hand, some fish samples still showed a quite low APC at T_2 (cod, salmon and pangasius). However, large fish tend to spoil more slowly than small fish, and also aquacultured fish may have a longer shelf life, when caught in ideal conditions than similar fish from wild catch (Huss, 1995). On the contrary, elasmobranch fish such as ray tend to spoil very quickly after catch, but this is always assumed to be mainly due to organoleptic changes (fast emission of ammonia) (Múgica et al., 2007). In this study, the

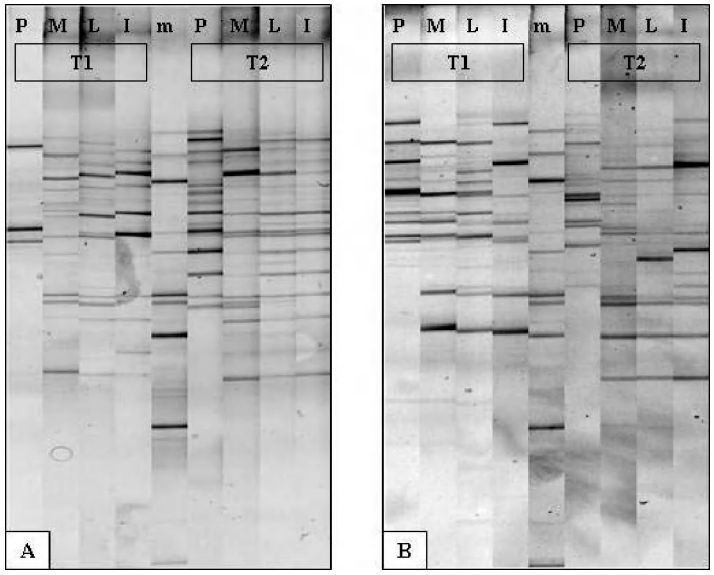

Fig. 3.3. PCR-DGGE - V3 profile of a plate swab of all media P (PCA), M (MA), L (LH), I (IA) from mackerel (A) and pangasius (B) at the day of arrival (T_1) and after 7 days of ice storage (T_2). An internal reference marker m was used for the comparison of different gels.

APC of ray was already quite high (6 $\log_{10}$ cfu/g on PCA or 7 $\log_{10}$ cfu/g on salt-containing media) at T_1.

The APC results confirm earlier publications indicating differences up to one log or more between PCA and LH or MA for marine fish species (Joffraud & Leroi, 2000; Kudaka et al., 2010; Van Spreekens, 1974). In this study, several fish samples (salmon, plaice, sole, sea bass and sea bream) have a difference of at least 1 log between PCA and LH/MA. The differences were noticed at T_1 as well as at T_2, indicating that fish quality analysis on PCA in fish auctions or supply center misses many microorganisms and underestimates the microbiological quality. A shift was also noticed during storage: in some cases, the number of microorganisms not growing on PCA increased, while in other fish samples those growing on PCA increased. This results in the differences in APC noticed at each sampling point. The microbiological growth during storage is mainly fish specific, depending on intrinsic and extrinsic characteristics of the fish and the handling procedures.

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

Replication showed many isolates that were unable to grow on one of the media, mostly on PCA. Many bacterial species on fresh fish are only able to grow on LH, MA or IA, but also a considerable part (21%) is unable to grow on one of those salt-containing media. After ice storage, however, the number of isolates not growing on PCA has increased to approximately 50% of the total number of isolates, while those unable to grow on LH, MA or IA have

decreased from 21 to 6%. This indicates that the microorganisms important for spoilage are mainly species needing those salt-containing media in order to be detected. The inability of microorganisms to grow could be caused by the absence of sodium chloride in PCA as previously noted in several studies (Boskou & Debevere, 1996; Liston, 1980; Vallé et al., 1998). The choice of growth medium may therefore be even more important during ice storage or at the end of shelf life than for (fresh) fish immediately after catch.

Identification of the non-growing colonies on one of the tested media at T_2 shows that some currently known marine SSOs are unable to grow on PCA, such as *Shewanella*. Especially *Shewanella putrefaciens* and *Shewanella baltica* are dominant microorganisms of ice-stored marine fish and are known as typical SSOs of marine fish species (Jorgensen & Huss, 1989; Koutsoumanis & Nychas, 1999; Vogel et al., 2005). All *Shewanella* species found, could be responsible for sensory spoilage since they are all capable of TMAO reduction and are important producers of H_2S. Although most of these species are described as nonhalophilic, this feature is strain specific and most species grow preferentially in the presence of 2% NaCl (Bowman et al., 1997; Satomi et al., 2007; Vogel et al., 2005). The *Shewanella* isolates in this study were all unable to grow on PCA.

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

Two less known genera in fish spoilage, *Pseudoalteromonas* (*Psa*) and *Psychrobacter* (*Psb*), were also unable to grow on PCA. Species of the genus *Pseudoalteromonas* were not detectable on PCA; they all require NaCl for growth. *Pseudoalteromonas* is a heat labile common marine microorganism. Species of this genus appear to be growing during storage of several fish and fishery products (Koutsoumanis & Nychas, 2000; Paarup et al., 2002; Romero et al., 2002; Rudi et al., 2004). *Pseudoalteromonas nigrifaciens* make up a large part of the microbiota for these fish samples after ice storage in this study (Table 3.3). In this study, also several species of the genus *Psychrobacter* were found. Some of the species

(*Psychrobacter proteolyticus* and *Psychrobacter cibarius*-like strains) were unable to grow on PCA, while others could only grow on PCA and not on the salt-containing media (*Psychrobacter fozii*, *Psychrobacter maritimus* and *Psychrobacter cibarius* strain). Except for *Psychrobacter cibarius*, for which growth has been reported to be stimulated by NaCl (Jung et al., 2005), the growth data of this study correspond with literature data (Bozal et al., 2003; Jung et al., 2005; Romanenko et al., 2004; Yoon et al., 2003). The genus *Psychrobacter* is currently not associated with major SSOs; *Psychrobacter immobilis* is a minor spoiler producing a musty off-odour commonly found on chilled fish (Gennari et al., 1999).

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

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

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

between the band patterns of cultivable microorganisms on the different growth media in order to have some insight in the similarity of species composition on these media. For most fish samples, the band pattern of PCA plate swabs is very different from those of the salt-containing media. The patterns of the salt-containing media, in contrast, resemble each other.

The APC analysis and replication technique results were in concordance with the differences in PCR-DGGE profiles of the fish samples. For mackerel, the differences in APC on the different media were not significant, but identification of the colonies not growing on PCA showed that species from the genera *Shewanella*, *Vibrio*, *Pseudoalteromonas* and *Psychrobacter* were present. On the other hand, species that only grow on PCA were also present. This explains the difference in PCR-DGGE pattern (no matching bands) of mackerel between PCA and the other media. For pangasius, the APC differences were small and PCR-DGGE band patterns on the different media were quite similar at T_1, but at T_2 differences in APC and PCR-DGGE pattern similarity increased possibly because genera such as *Shewanella* and *Pseudoalteromonas* were found which only grow on salt-containing media.

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

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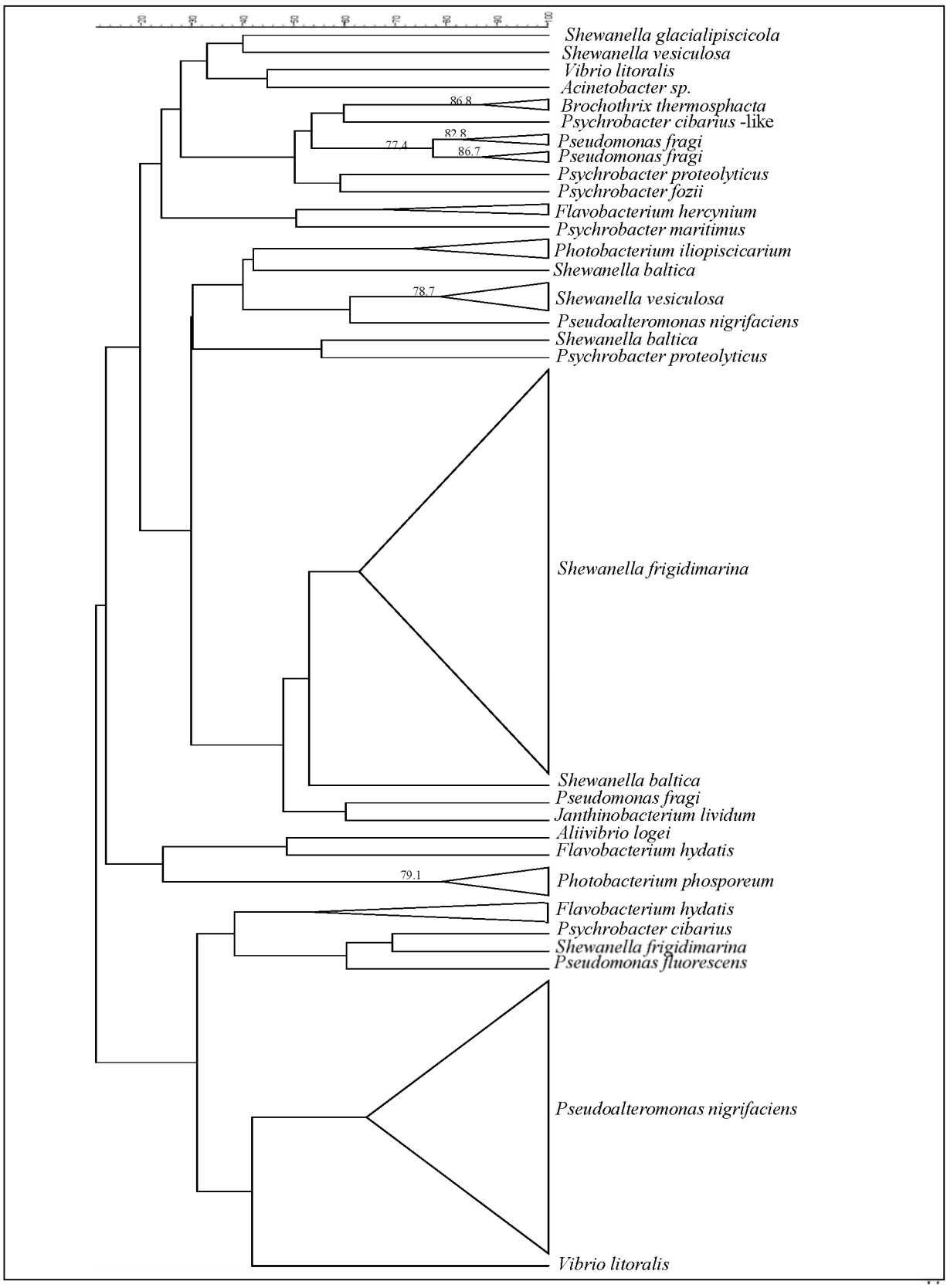
this manuscript, we wish to thank Miriam Levenson. Last we want to express our gratitude to our colleagues at ILVO's Plant Sciences Unit, for performing the sequencing experiments.

Suppl. Fig. 3.1. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-rep-PCR fingerprints. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentages values of the Pearson correlation coefficient. Relevant cophenetic values (above 75%) are shown. Identification of isolates was performed by 16S rRNA gene or gyrB gene sequencing (summarized in Table 3.3).



PART II

BROWN SHRIMP (*CRANGON CRANGON*): THE DOMINANT MICROBIOTA AND THEIR SPOILAGE POTENTIAL

Preamble

Brown shrimp without preservatives are a local delicacy in Belgium. However, these are extremely sensitive to spoilage possibly due to their high NPN fraction, which is easily metabolized by microorganisms. Since spoilage of seafood is mainly caused by microbial growth and activities, it's important that the microorganisms responsible for the deterioration by off-odours and off-flavours are identified. These microorganisms are called SSOs and are not only seafood specific but also depend on the storage conditions. In international literature, research about the microbiota present during spoilage on brown shrimp is rare, incomplete and out dated. In order to identify the SSOs of brown shrimp without preservatives, the dominant microbiota on shrimp are detected via traditional and molecular (culture dependent and independent) methods in chapter 4. Subsequently is the dominant microbiota identified via 16S rRNA and gyr*B gene sequencing. In chapter 5, the spoilage potential of several isolates is determined and quantified via mass spectrometry analyses.*

CHAPTER 4

MOLECULAR IDENTIFICATION OF THE MICROBIOTA OF PEELED AND UNPEELED BROWN SHRIMP (*CRANGON CRANGON*) DURING STORAGE ON ICE AND AT 7.5°C

Redrafted from:

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F. and Vlaemynck, G. 2011. Molecular identification of the microbiota of peeled and unpeeled brown shrimp (*Crangon crangon*) during storage on ice and at 7.5°C. Food Microbiology. Submitted for publication.

Chapter 4. Molecular identification of the microbiota of peeled and unpeeled brown shrimp (*Crangon crangon*) during storage on ice and at 7.5°C

Abstract

The dominant microbiota of brown shrimp (*Crangon crangon*) were systematically identified during storage under different conditions. Freshly caught shrimp were processed on board the fishing vessel under the best possible hygienic conditions (IDEAL), unpeeled and manually (sterile) peeled, then stored on ice and at 7.5°C until microbiologically spoiled. Results were compared with industrially processed (INDUSTRIAL) shrimp. Isolates grown on various media were identified by 16S rRNA and *gyr*B gene sequencing. We examined the total microbiota and microbiological population shifts of shrimp under various storage conditions using denaturant gradient gel electrophoresis (PCR-DGGE). The microbiota differed somewhat during storage and among the various storage conditions; however, members of the genera *Psychrobacter* and *Pseudoalteromonas* were found to dominate the microbiota of all shrimp samples regardless of processing procedures or storage conditions. Most isolates could be identified by *gyr*B gene sequencing as *Psychrobacter immobilis* or *Psychrobacter cibarius*. Also *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas elyakovii* or *Pseudoalteromonas paragorgicola* dominated the microbiota of brown shrimp during storage. Also species from the genera *Planococcus*, *Exiguobacterium*, *Carnobacterium*, *Pseudomonas*, *Chryseobacterium* and *Staphylococcus* were detected during storage of brown shrimp.

Culture-dependent and culture-independent PCR-DGGE analysis produced different results in band patterns. Both methods are therefore required to accurately identify the microbiota and bacterial population shifts on seafood during storage.

I. Introduction

The brown shrimp (*Crangon crangon*) is a typical Belgian seafood product, especially machine-peeled and unpeeled brown shrimp, processed without preservatives and other additives, are a local delicacy. As described in chapter 2, the shrimp landed in Belgian harbours by Flemish fishermen are artisanally processed on board the vessels. Immediately after being caught, the shrimp are cooked for a short time, resulting in a decrease of the bacterial count. After cooking, the shrimp are cooled either by rinsing with clean seawater or

exposing them to the air, then stored in large boxes with ice in a cooled space below deck. The shrimp are landed no more than 24 hours after being caught.

Like other fresh and lightly preserved seafood, brown shrimp are prone to rapid spoilage due to their high number of non-protein nitrogenous compounds which are easily metabolised by microorganisms (Liston, 1980). The shrimp's shelf life can be extended by adding preservatives (commonly 0.6% benzoic acid and 0.6% sorbic acid). However, consumer pressure to reduce the use of preservatives may influence this practice.

The aim of this study was to develop a molecular identification technique for the dominant microbiota present on brown shrimp stored without preservatives or other additives either on ice or at a temperature of 7.5 ± 0.5°C (Marklinder et al. 2004). This temperature is also in agreement with the mean temperature (6.7 ± 2.7°C) in consumer's fridges as determined by a study performed (WIV 2006). The microbiota of shrimp processed and stored under several circumstances was identified to species level. The total microbiota was compared between the store-bought (INDUSTRIAL) and self-processed (IDEAL) samples, together with microbiological shifts observed under different processing and storage conditions, such as manual or machinal peeling and storage on ice versus storage at 7.5°C. Those analyses were made using denaturant gradient gel electrophoresis (PCR-DGGE).

II. Materials and methods

A scheme of the storage conditions and performed analyses of the shrimp sampled on board of a fishing vessel under the best possible hygienic conditions (further mentioned as IDEAL shrimp) and the shrimp purchased at the dock, which had been processed according to normal industrial procedures (further mentioned as INDUSTRIAL shrimp) is shown in Fig 4.1.

2.1. Sampling of shrimp on board a fishing vessel under the best possible hygienic conditions (IDEAL) and during storage at different temperatures

The shrimp were caught using a beam trawler in March 2008 and processed under good hygienic conditions on board. Specifically, the artisanal processing procedures described above were duplicated but conditions were optimized by 1) minimal cross-contamination between raw and cooked material, 2) (fast) cooling by air exposure after cooking, and 3) continual ice storage in a large amount of fresh ice on the fishing vessel, during transport, and during storage at the lab. The shrimp were machine washed and hand sorted immediately after being caught. After sorting, one sample of raw shrimp was aseptically placed in a sterile stomacher bag. Another sample was cooked according to normal Belgian fishing procedures

(2 to 4 minutes at 86 to 98°C) in clean seawater with some extra salt added. Cooked shrimp never came in contact with material used for raw shrimp. After air-cooling, the cooked shrimp were aseptically placed in a sterile stomacher bag. Both samples were immediately put on ice for transport to the laboratory. No additives or preservatives were added. Twenty-four hours after catch (T_0), the samples arrived at the laboratory. From the sample of cooked shrimp, half was manually peeled under nearly sterile conditions (wearing sterile gloves under a laminar flow hood, with minimal cross-contamination between peel and flesh). Half of the peeled and unpeeled shrimp were stored in sterile bags on ice (0 ± 0.5°C), while the other half was stored in the refrigerator at 7.5 ± 0.5°C. Microbiological analyses were performed at regular time intervals, i.e., at arrival (T_0), after 7 days (T_1) and after 13 days (T_2). The shrimp were stored until microbiologically spoiled (APC > 10^7 cfu/g).

2.2. Sampling of purchased (INDUSTRIAL) shrimp and during storage on ice

For purposes of comparison, shrimp without preservatives were purchased at the dock in a small fish store that specialized in brown shrimp in June 2010. These samples had been processed according to normal industrial procedures. These shrimp had been caught, sorted, cooked on board, and machine cooled using clean cold seawater. Upon landing, the shrimp were machine-sorted and sold either unpeeled or machine-peeled. Both preparations (peeled and unpeeled) were used as comparison with the hygienically prepared, ice-stored sample described above. These purchased samples of peeled and unpeeled shrimp were put on ice for transport to the lab and during storage at the lab. Microbiological analyses were performed at arrival (T_0) and after 7 days of storage on ice (T_1).

2.3. Cultivation of microorganisms of IDEAL shrimp

For the IDEAL shrimp samples, several growth media were used to obtain a complete view of the microbiota of raw, cooked unpeeled and peeled shrimp during storage on ice and at 7.5°C. Three media were used for the total 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 H_2S (black colonies) were enumerated on Lyngby iron agar (IA; Atlas, 2006). The composition of LH and IA medium is described in chapter 3 (2.2.).

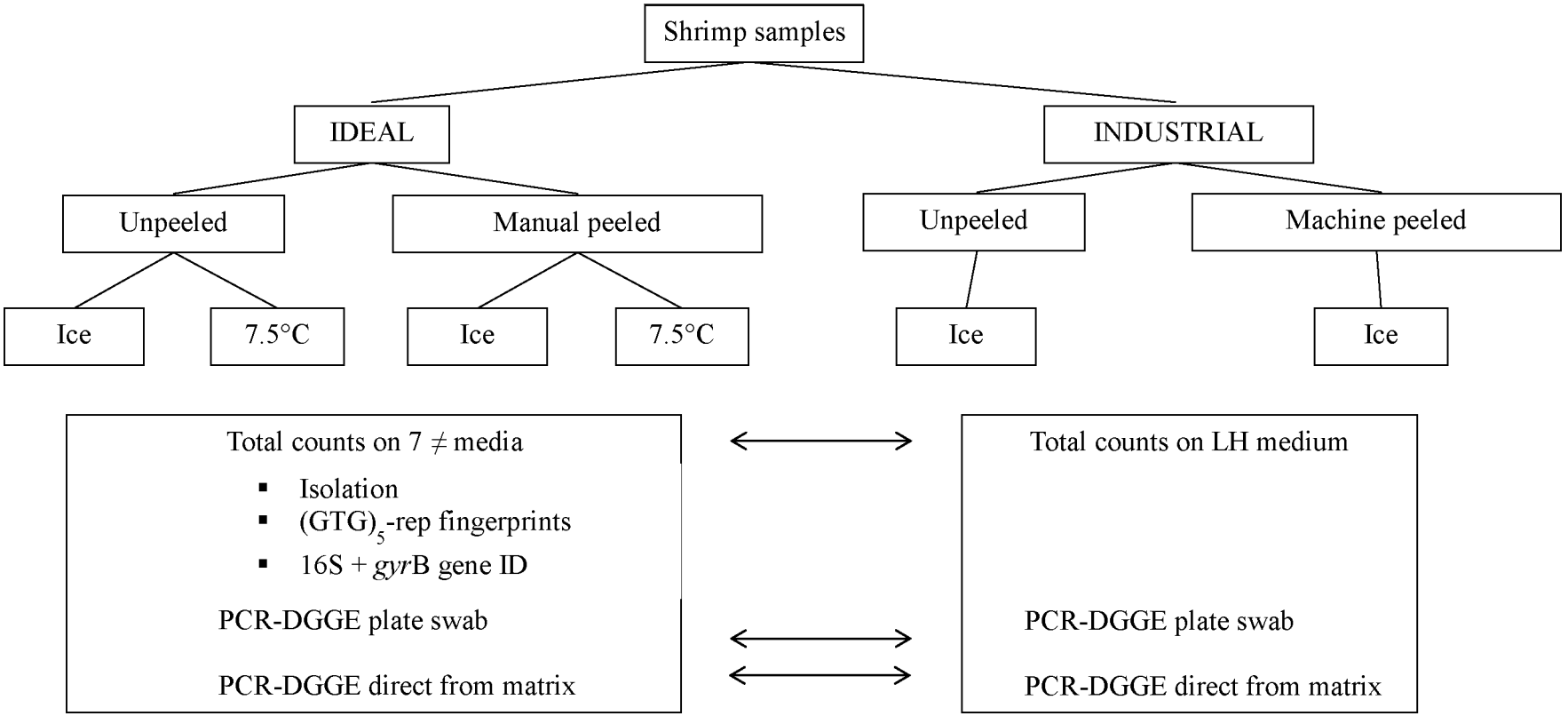


Fig 4.1. Scheme of the storage conditions (unpeeled, peeled, stored on ice or at 7.5°C) and performed analyses of the shrimp sampled on board of a fishing vessel under the best possible hygienic conditions (IDEAL) and the shrimp purchased at the dock, processed according to normal industrial procedures (INDUSTRIAL). LH= modified Long and Hammer medium (Van Spreekens, 1974).

The microbiological analyses of purchased shrimp during storage on ice were only performed on LH medium, since this medium appears to be the best growth medium for enumeration of the microbiota of fresh seafood as observed in chapter 3.

For the microbiological analysis, 10 g shrimp was transferred aseptically to a stomacher bag, 90 ml maximum recovery diluent (Oxoid) was added and the mixture was homogenized for 2 min. A part of this homogenized mixture (50 ml) was collected and stored at -20°C for the culture-independent PCR-DGGE analysis (2.5.2.). 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. Duplicates were made for every sample. After incubation, all colonies were counted. After enumeration, the duplicates of PCA and LH plates were used for the plate swabs for cultivation-dependent PCR-DGGE analysis (2.5.1.).

2.4. Identification of the isolates from shrimp processed under the best possible conditions

2.4.1. DNA extraction

A selection of 390 isolates with different colony morphology were selected from PCA, MA, IA, LH and CFC media. Of those 390 isolates, 210 were picked from cooked shrimp samples. These isolates were purified and DNA extraction was performed as described in chapter 3 (2.4.1.). The DNA was stored at -20°C in HPLC water.

2.4.2. Rep-PCR

The purified strains were clustered based on their $(GTG)_5$ rep-fingerprint. The PCR was performed as described in chapter 3 (2.4.2.). PCR products were size separated in a 1.5% agarose gel in 1x TBE buffer at 120V for 4h. 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 performed in chapter 3 (2.4.2.).

2.4.3. Identification of the microbial isolates by sequence analysis

Fifty-two representatives from the $(GTG)_5$ clusters generated from isolates from the cooked unpeeled and peeled shrimp were selected for identification. A 1500 bp fragment of the 16S rRNA gene and the *gyr*B gene was amplified as previously described in chapter 3 (2.4.3.). 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) were performed as described in Broekaert et al. (2011). 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). For isolates of the genera *Psychrobacter* and *Pseudoalteromonas*, the complete 16S rRNA (16F27 and 16R1522 primer pair) and *gyr*B gene sequences were sequenced and 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) web search (http://www.ebi.ac.uk/tools/fasta33/nucleotide.html). When similarity value was low, phylogenetic trees were constructed as previously described in Broekaert et al. (2011). The *gyr*B sequences of the type strains were submitted to the EMBL nucleotide sequence database (accession numbers FR668560 to FR668582).

2.5. Observation of the microbiological community using PCR-DGGE analysis

PCR-DGGE analysis was performed on the IDEAL shrimp sampled in March 2008 and the INDUSTRIAL shrimp purchased in June 2010. Both sets of samples were unpeeled and peeled. Since differences between culture-dependent and independent PCR-DGGE analyses are often observed (Ampe et al., 1999), both methods were performed and results compared.

2.5.1. Sample preparation for culture-dependent PCR-DGGE analysis

Complete plate swabs of the PCA (only for the IDEAL samples) and LH growth media were taken immediately after enumeration. The dilution used for the plate swab depended on the APC of the sample and counts between 30 and 150 were chosen. The pellets were washed twice with 1x PBS (137mM NaCl, 2.7mM KCl, 0.9mM KH_2PO_4 and 6.4mM Na_2HPO_2 (pH7.4)) then stored at -20°C until use. A DNA extraction was performed using the Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. This DNA was stored at -20°C until further usage by PCR-DGGE.

2.5.2. *Sample preparation for culture-independent PCR-DGGE analysis*

A culture-independent DNA extraction from the shrimp sample was performed according to Rudi et al. (2004). Briefly, the method consisted of 1:10 dilution of the shrimps in peptone water, which were then homogenized for 2 min before freezing 50 ml of each suspension. For bacterial extraction, the tubes were thawed and diluted 1:2 with peptone water before centrifugation for 2 min at 70 g (Sorvall RC 26 plus, Thermo Scientific). The supernatant was removed and collected until approximately 10 ml was left. 90 ml peptone water was added to the fish suspension and the centrifugation was repeated. The supernatant was added to the supernatant from the first centrifugation, then the suspension was centrifuged for 15 min at 14500 g. Pellets were resuspended in 10 ml TE-buffer pH 8 (10 mM Tris–HCl and 1 mM EDTA) then centrifuged for 10 min at 8500 g. Pellets were resuspended in 5 ml TE-buffer and DNA was purified using the Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.5.3. *PCR-DGGE analysis and identification of the bands*

The PCR for PCR-DGGE analysis was performed as described in chapter 3 (2.5.). PCR-DGGE analysis of the PCR amplicons was performed on the DCode Universal Mutation Detection system (Biorad) as described by Muyzer et al. (1993). Samples were applied to an 8% (w/v) polyacrylamide gel in 1x TAE buffer (Invitrogen). Optimum parallel electrophoresis experiments were performed at 60°C using gels containing a 40 to 60% urea-formamide denaturing gradient. Electrophoresis was performed at a constant voltage of 45V for 14h. After separation, the gels were incubated for 20 minutes in 1x TAE pH 8 containing Sybr Gold staining and photographed with UV transillumination (G:BOX, Syngene). Bands were detected manually and analysed using Bionumerics version 6.5 software (Applied Maths).

For interpretation of the band patterns and normalization of the gels, an internal marker was made including pure strains of the genera *Pseudoalteromonas* and *Psychrobacter*. As template for the PCR-DGGE-PCR analysis, 10ng DNA of several identified microorganisms was pooled. This mixture of pure strains was used for the PCR as described above and was run on PCR-DGGE with the samples.

Eleven bands were selected from the gels for further identification. The selected PCR-DGGE fragments were punched from the gel. The pieces were transferred into 20µl of sterile water and were incubated overnight at 4°C. Two µl of the eluted DNA were used for

reamplification using the same protocol as described above and the PCR products were verified for purity using PCR-DGGE. The corresponding bacterial community PCR-DGGE profile was used as control in an adjacent lane. Products that migrated as a single band, located at the same position as the control, were purified using the High Pure PCR product purification kit (Roche) according to the manufacturer's protocol and sequenced using the primer UN357f without GC clamp. Identification of the nearest phylogenetic neighbours was performed using a nucleotide similarity search against the *Eztaxon* and FASTA database as described above.

III. Results

3.1. Microbiological analysis of IDEAL shrimp

Several media (general and specific) were used to obtain a complete view of the total microbiota on IDEAL shrimp sampled on board. Table 4.1 shows the microbiological counts on all media for these raw shrimp after manual sorting and for cooked unpeeled shrimp. Fig. 4.2 (A: unpeeled, B: peeled) shows the microbiological counts for cooked shrimp during storage on ice and at 7.5°C. Since the APC on MA and LH were more or less similar, only the results on LH are discussed below.

Table 4.1. Microbiological counts on all growth media in $\log_{10}$ cfu/g of raw shrimp and of cooked unpeeled shrimp sampled on board and processed under the best possible conditions at T_0. /: not performed. 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).

Microbiological counts of shrimp ($\log_{10}$ cfu/g)						
	Shrimp processed under best possible conditions at T_0		Purchased shrimp at T_0 and T_1			
			Unpeeled		Peeled	
Medium	**Uncooked**	**Cooked**	**T_0**	**T_1**	**T_0**	**T_1**
PCA	4.9	2.9	/	/	/	/
LH	6.5	3.5	5.0	8.3	4.1	7.6
MA	6.5	4.1	/	/	/	/
CFC	4.6	2.7	/	/	/	/
MRS	2.6	2.2	/	/	/	/
VRBGA	1.0	<10	/	/	/	/
IA	2.2	<10	/	/	/	/

3.1.1. Effect of cooking on the microbiota of unpeeled IDEAL shrimp

The brief cooking of the shrimp decreased the total microbiota (Table 4. 1) by 2 log (PCA) to 3 log (LH). On *Pseudomonas*-specific (CFC) medium, a 2 log reduction was observed. The

number of LAB (MRS) was more or less equal before and after cooking. *Enterobacteriaceae* (VRBGA) were absent in 10 g of shrimp during most of the study. The number of sulphide producers (IA (H_2S)) decreased by 2.2 log to below the detection level.

3.1.2. Microbiological analysis of unpeeled IDEAL shrimp stored on ice

The microbiological counts of cooked unpeeled shrimp are shown below (Fig 4.2A). On freshly cooked unpeeled shrimp on ice at arrival in the laboratory (T_0) an APC of 2.9 (PCA) or 3.5 (LH) log cfu/g was counted. During storage on ice, the microbiota increased rapidly; after 7 days (T_1) a 2.5 (PCA) to 2.6 (LH) log increase was observed. After 13 days (T_2), an APC of 7.4 (PCA) or 8.8 (LH) was reached and the shrimp were considered to be microbiologically spoiled (APC: > 7 $\log_{10}$ cfu/g). On CFC, the numbers of *Pseudomonas* spp. increased rather slowly during storage on ice; at T_1 the number increased by 0.8 log, at T_2 by 3.2 log. The number of LABs (MRS) was rather low and stayed equal during the whole study. No *Enterobacteriaceae* were counted on VRBGA in 10 g of unpeeled shrimp during storage on ice. Sulphide producers were absent during the storage on ice until end of storage (T_2), when a 1.5 log increase was observed.

3.1.3. Microbiological analysis of manually peeled IDEAL shrimp during storage on ice

After arrival at the laboratory (T_0), half of the cooked shrimp were peeled manually under sterile conditions. In general, fewer microbiota were observed than on unpeeled shrimp during ice storage (Fig 4.2B). At T_1, a -0.8 (LH) to -1.4 (PCA) log difference was observed as compared to the unpeeled shrimp stored under identical conditions. At T_2, a -1 log difference with unpeeled shrimp was observed on PCA, whereas on LH the difference was within a standard error range of 0.5 log (-0.3 log). For *Pseudomonas* spp. (CFC), no difference between peeled and unpeeled shrimp was observed at T_1. At T_2 however, a difference of -1.1 log was observed between peeled and unpeeled shrimp. Peeling also decreased the number of LAB by -1.4 log at T_1 and -0.6 log at T_2 compared with unpeeled shrimp. *Enterobacteriaceae* (VRBGA) were absent in 10 g of peeled shrimp during storage on ice. Peeling the shrimp also reduced the number of sulphide producers (IA) by 1.5 log at T_2 to below the detection level as compared with unpeeled shrimp.

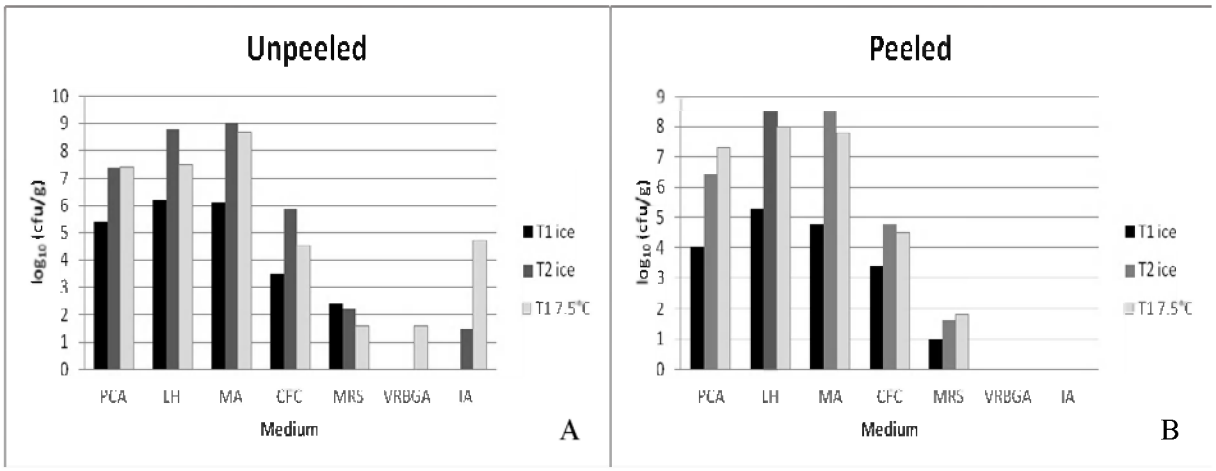


Fig 4.2. Microbiological counts (log_{10} cfu/g) on all growth media of unpeeled (A) and manual peeled (B) shrimp sampled on board processed under the best conditions at T_1 (7days of storage) and T_2 (13 days of storage) on ice and at 7.5°C. PCA= plate count agar, LH= modified Long and Hammer medium (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). The standard error between duplicate plates on the different media were added.

3.1.4. Effect of storage at different temperatures on the microbiota on IDEAL shrimp

In general, storage at 7.5°C revealed a much higher microbiological contamination on both the unpeeled (Fig 4.2A) and the peeled (Fig 4.2B) shrimp as compared to shrimp stored on ice. At T_1, the shrimp stored at 7.5°C were considered spoiled due to a very high APC (> 7 log_{10}) and an strongly disagreeable odour. The unpeeled shrimp at 7.5°C showed a difference of 1.4 (LH) to 2 (PCA) log as compared with those stored on ice (Fig 2A). A 1 log difference of *Pseudomonas* spp. (CFC) was also observed. Some *Enterobacteriaceae* (VRBGA: 1.6 log) and a high number of H_2S producers (IA: 4.7 log) were able to grow very well at this temperature, while they were absent in 10 g of shrimp on ice at T_1. On the contrary, the number of LAB (MRS) during storage at a higher temperature appeared somewhat (0.8 log) lower than on ice.

The difference in bacterial numbers for peeled shrimp (Fig 4.2B) between ice storage and storage at 7.5°C was even larger than for unpeeled shrimp, especially for the general media (PCA and LH). When stored at 7.5°C, the difference in APC as compared with ice storage was approximately 3 log. The number of *Pseudomonas* spp. on CFC medium increased by 1 log when stored at 7.5°C, while LAB (MRS) showed an increase of 0.8 log. In contrast to the unpeeled shrimp stored at high temperature, no *Enterobacteriaceae* or sulphide producers were detected in 10 g of peeled shrimp at T_1.

3.2. Microbiological analysis of INDUSTRIAL shrimp stored on ice

Enumeration of the APC on purchased shrimp as grown on LH was much higher than from IDEAL shrimp (Table 4.1). At T_0, the APC was already 5 $\log_{10}$ cfu/g for unpeeled shrimp and 4.1 $\log_{10}$ cfu/g for machine peeled shrimp. After 7 days of ice storage (T_1), the purchased shrimp were considered to be microbiologically spoiled with an APC of 8.3 $\log_{10}$ cfu/g (unpeeled) and 7.6 $\log_{10}$ cfu/g (peeled).

3.3. Molecular identification of the isolates of IDEAL shrimp

In total, 390 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 isolates showed great variety, with some large clusters of related isolates (similarity >64%). Identification of those large clusters was important as those are part of the most abundantly recovered microbiota. Additionally, representatives of all isolates present at T_2 were identified.

Of the isolates, 180 came from raw shrimp. Some isolates could be identified based on comparing their rep-profile with the profiles of isolates from cooked shrimp. Those only present on raw shrimp were not identified, given that most shrimp are usually cooked immediately after catch.

The remaining 210 isolates were collected from cooked unpeeled (129) and peeled (81) shrimp during storage at all time points during storage on ice and at 7.5°C. From the $(GTG)_5$-rep-fingerprints, 52 representatives were selected and identified 158 of the 210 isolates to genus level based on their partial 16S rRNA gene sequence. Most of the identified isolates belonged to the genera *Psychrobacter* (40%) and *Pseudoalteromonas* (34%). Other genera present during storage were *Planococcus*, *Loktanella*, *Pseudomonas*, *Exiguobacterium* and *Chryseobacterium* (Table 4.3). However, these genera were only present in 1-2 isolates per genus. The remaining 52 of the 210 isolates either had an unique $(GTG)_5$-rep-fingerprint or did not grow out during further storage and were therefore not identified.

3.3.1. Identification of the dominant microbiota on unpeeled IDEAL shrimp

One hundred twenty-nine isolates of unpeeled shrimp were collected during storage on ice or at 7.5°C. From the $(GTG)_5$-rep clusters, 26 representatives were selected and identified 88 of the 129 isolates to genus level based on their partial 16S rRNA gene sequence. Immediately after cooking (T_0), isolates from the genera *Psychrobacter*, *Pseudoalteromonas*, *Loktanella* (one isolate) and *Planococcus* (one isolate) were found. During storage on ice (at T_1 and T_2),

the microbiota on shrimp consisted only of *Psychrobacter* and *Pseudoalteromonas* species. When stored at 7.5°C, *Psychrobacter* species and one *Exiguobacterium* isolate were identified.

3.3.2. *Identification of the dominant microbiota on manually peeled IDEAL shrimp*

Eighty-one isolates were collected from the manually peeled shrimp during storage on ice and at 7.5°C. From the $(GTG)_5$-rep fingerprints, 25 representatives were selected and identified 63 of the 81 isolates were identified to genus level. During storage, two genera, *Psychrobacter* and *Pseudoalteromonas*, were found abundantly on peeled shrimp. On ice a *Pseudomonas* isolate was found at T_1. When stored at 7.5°C *Psychrobacter* and *Pseudoalteromonas* species were present in high amounts; one isolate of *Chryseobacterium* and one *Planococcus* isolate were also found.

3.3.3. *Species identification of the isolates belonging to the genera Psychrobacter and Pseudoalteromonas*

After cooking, the most abundant genera on shrimp were *Psychrobacter* and *Pseudoalteromonas*, independent of the storage conditions. Partial 16S rRNA gene sequencing could identify the isolates to genus level only, since it resulted in species complexes for those two genera. Therefore, 44 isolates with different $(GTG)_5$-rep fingerprints were selected for further identification to species level using the nearly complete 16S rRNA and *gyr*B gene sequence. Supplementary Table 4.1 (*Psychrobacter*) and supplementary Table 4.2 (*Pseudoalteromonas*) show the tentative identifications of the isolates based on similarity searches against *Eztaxon* (16S) and FASTA (*gyr*B) web searches. Between both genes, discrepancies in identification were often found. The phylogenetic trees based on the *gyr*B gene (1500 bp) sequence are shown in Figs. 4.3 and 4.4; the phylogenetic tree for *Psychrobacter* based on the full 16S sequence is shown in Suppl Fig 4.1.

According to the phylogenetic tree based on the full 16S rRNA gene sequences of *Psychrobacter* isolates (Suppl. Fig 4.1.), 21 isolates were assigned to *Psychrobacter cibarius*, one isolate (G12) was identified as *Psychrobacter fozii*, one (G65) as *Psychrobacter marincola* and six isolates remained unknown at species level. Fig 4.3 shows the phylogenetic clustering of 29 isolates of the genus *Psychrobacter* based on the *gyr*B gene. Most isolates (21) are most closely related to *Psychrobacter immobilis* or *Psychrobacter cibarius*, with two isolates clearly identified as *Psychrobacter cibarius* (2G40 and 1G232). For most of the other isolates within this cluster, unambiguous species identification was not possible. One isolate

(1G194) was most related to *Psychrobacter okhotskensis* and one to *Psychrobacter marincola* (G65). The same six isolates (G30, G34, G56, G67, G70 and G71) which could not be identified based on their full 16S rRNA gene sequence remained unknown to species level using *gyr*B as well.

For the genus *Pseudoalteromonas*, identification based on the full 16S rRNA gene did not seem possible, since several type strains seemed to have the same sequence (i.e., *Pseudoalteromonas distincta*, *Pseudoalteromonas paragorgicola* and *Pseudoalteromonas. elyakovii*). The isolate 2G68 was most closely related to *Pseudoalteromonas translucida* and *Pseudoalteromonas antarctica* based on the full 16S sequence. The isolates 1G272 and 1G161 were closely related to all other isolates and the type strains of *Pseudoalteromonas distincta*, *Pseudoalteromonas paragorgicola* and *Pseudoalteromonas elyakovii* based on their full 16S sequence, but they were separated in genetic distance from the other isolates based on *gyr*B. Fig. 4.4 shows the phylogenetic tree based on the *gyr*B gene including 15 isolates. Eleven of those isolates were identified as *Pseudoalteromonas nigrifaciens*. One was closely related to *Pseudoalteromonas paragorgicola* (2G68) and two (1G272 and 1G161) were closely related to *Pseudoalteromonas elyakovii* and *Pseudoalteromonas paragorgicola*. One isolate (G41) remained unidentified to species level.

3.4. Bacterial population dynamics during storage of shrimp as revealed by PCR-DGGE analysis

We used PCR-DGGE analysis to investigate the microbiological diversity and dynamics of the dominant microbiological communities on brown shrimp during storage. PCR-DGGE analysis was performed on plate swabs from PCA and from LH (Fig 4.5) and from DNA extracted directly from the shrimp matrix (Fig 4.6). The appearance or disappearance of amplicons (bands) in the PCR-DGGE pattern indicates the possible detectability of the dominant microbiota present on the various growth media and indicates important shifts in the microbiological community during storage on ice or at 7.5°C.

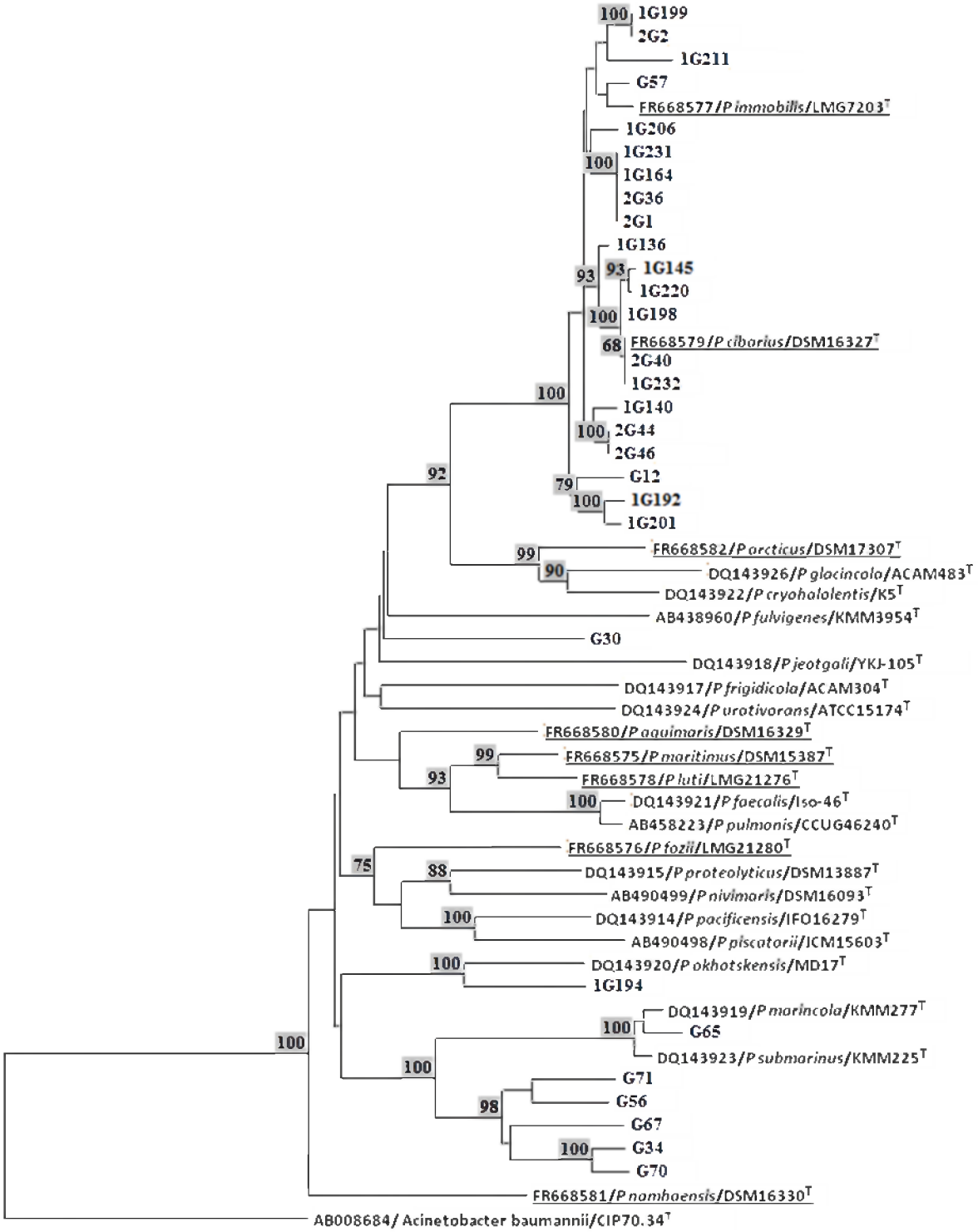


Fig 4.3. Phylogenetic tree of the genus *Psychrobacter*, with the isolates from brown shrimp included, based on the *gyr*B gene sequence (1500bp). The type strains which were sequenced and submitted to the EMBL nucleotide sequence database are underlined. The tree was constructed with the neighbour-joining method. Genetic distances were calculated by the Jukes & Cantor model. The scale shows the genetic distance of 0.02. The percentages on the nods give the bootstrap values (on 1000 bootstrapped trees). Only values above 65% are shown. *Acinetobacter baumannii* was used as outgroup.

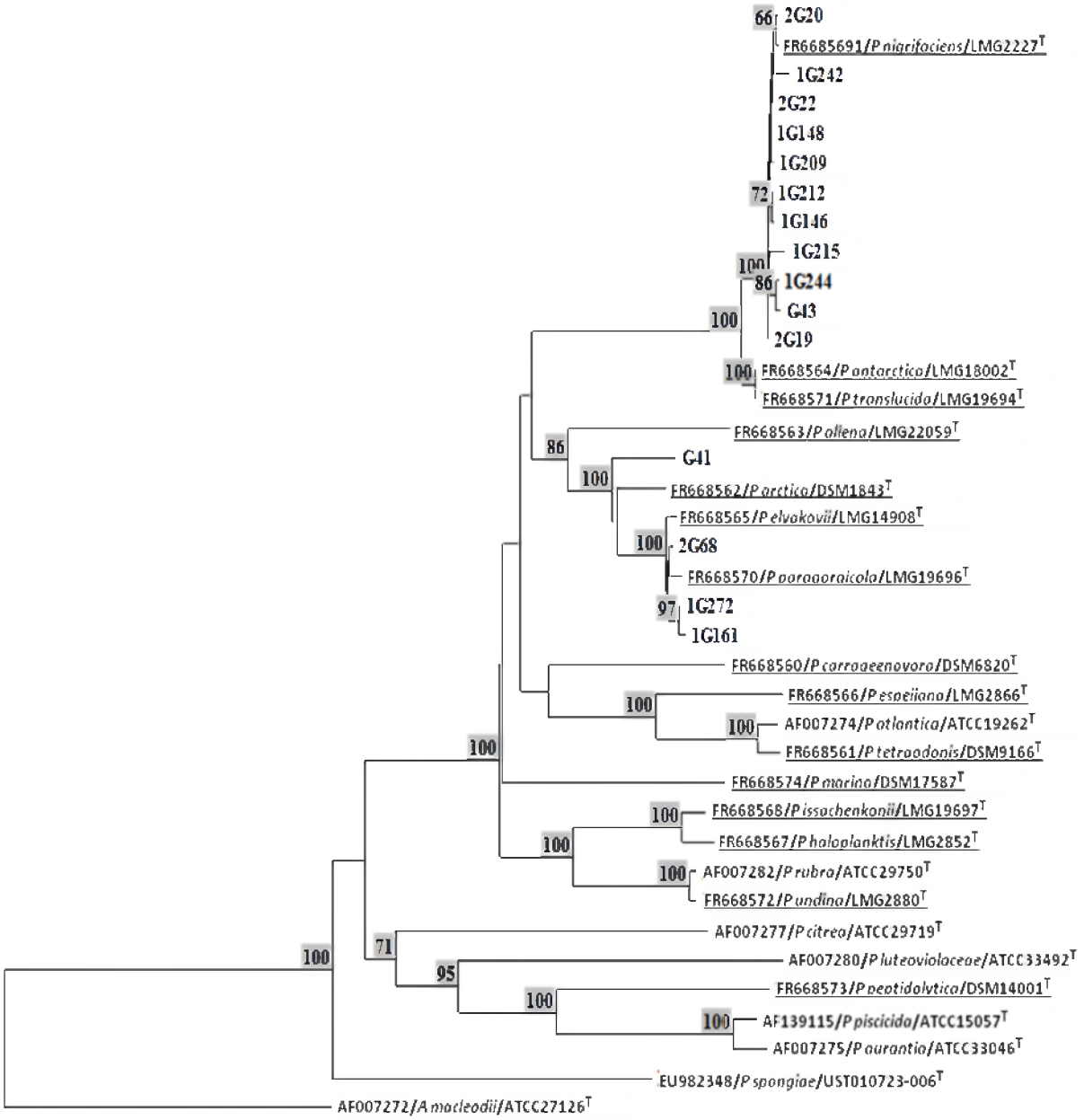


Fig 4.4. Phylogenetic tree of the genus *Pseudoalteromonas,* with the isolates from brown shrimp included, based on the *gyr*B gene sequence (1500bp). The type strains which were sequenced and submitted to the EMBL nucleotide sequence database are underlined. The tree was constructed with the neighbour-joining method. Genetic distances were calculated by the Jukes & Cantor model. The scale shows the genetic distance of 0.02. The percentages on the nods give the bootstrap values (on 1000 bootstrapped trees). Only values above 65% are shown. *Alteromonas macleodii* was used as outgroup.

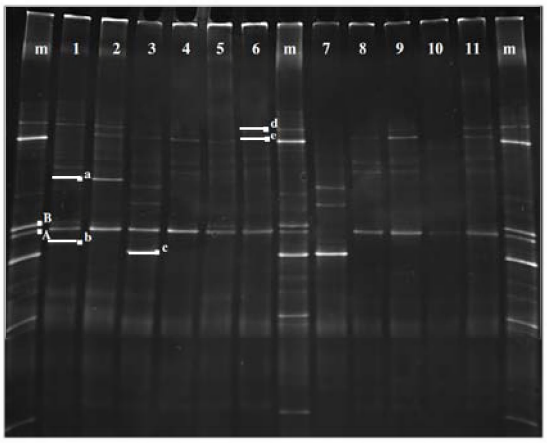


Fig 4.5. PCR-DGGE pattern from the microbiota of cooked shrimp swabbed from modified Long and Hammer medium (LH). m= marker, A= *Pseudoalteromonas*, B= *Psychrobacter*; 1-4 and 7-9: samples from shrimp processed under best conditions; 5-6 and 10-11: samples from purchased shrimp. 1 and 5= unpeeled shrimp stored on ice at T_0, 2 and 6= unpeeled stored on ice at T_1, 3= unpeeled stored at 7.5°C at T_1, 4= unpeeled stored on ice at T_2. 7 and 11= peeled stored on ice at T_1, 8= peeled stored at 7.5°C at T_1, 9= peeled stored on ice at T_2, 10= peeled stored on ice at T_0. The small letters correspond to those in table 4.2.

The internal marker with *Pseudoalteromonas* and *Psychrobacter* included several isolates of those genera and showed (Figs 4.5 and 4.6) that in many samples both genera were present during storage on ice as well as at 7.5°C. The presence of *Psychrobacter* was mostly observed on PCA plate swabs, while *Pseudoalteromonas* was only observed on LH plate swabs. Table 4.2 shows the identification of the excised PCR-DGGE bands. Several genera, namely *Psychrobacter* sp. (PCA: band k), *Carnobacterium* sp. (PCA), *Bacillus* sp. (LH), and *Pseudoalteromonas* sp. (LH) could be recovered from only one of the growth media.

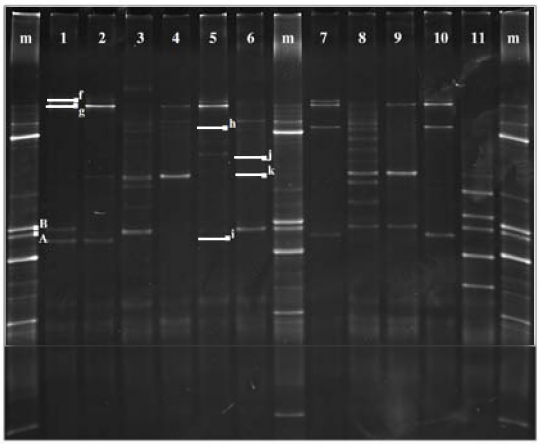


Fig 4.6. PCR-DGGE pattern from the microbiota of cooked shrimp from DNA extracted without cultivation direct from the shrimp matrix. m= marker, A= *Pseudoalteromonas*, B= *Psychrobacter*; 1-4 and 7-9: samples from shrimp processed under the best possible conditions; 5-6 and 10-11: samples from purchased shrimp. 1 and 5= unpeeled shrimp stored on ice at T_0, 2 and 6= unpeeled stored on ice at T_1, 3= unpeeled stored at 7.5°C at T_1, 4= unpeeled stored on ice at T_2. 7 and 11= peeled stored on ice at T_1, 8= peeled stored at 7.5°C at T_1, 9= peeled stored on ice at T_2, 10= peeled stored on ice at T_0. The small letters correspond to those in table 4.2.

Some differences were observed between the PCR-DGGE patterns obtained by plate swabs and via direct DNA extraction from the matrix (Fig. 4.5 and 4.6). The genera corresponding with the excised band a and bands c to e were only visualized via plate swabs. On the other hand, the genera corresponding to bands g (*Exiguobacterium* sp.) and i (*Staphylococcus* sp.) were only observed via direct DNA extraction.

Further differences were observed in PCR-DGGE patterns between unpeeled and peeled shrimp. According to the identified bands the species of the genus *Pseudoalteromonas* corresponding with bands d and e (Fig. 4.5) dominated the microbiota of unpeeled shrimp and were not observed on peeled shrimp (Fig. 4.6). Marker-based identification, however, showed that most samples did contain *Pseudoalteromonas* species.

The PCR-DGGE patterns from the INDUSTRIAL shrimp showed that the genera *Pseudoalteromonas* and *Psychrobacter* were both present. They were also found on the IDEAL shrimp. Other bands, especially from the DNA directly extracted from the matrix, also showed some similarity between both shrimp samples. Species from the genus *Staphylococcus* (band i) were only found on INDUSTRIAL shrimp and not on the IDEAL shrimp. *Pseudoalteromonas arctica* (band e) was only found on INDUSTRIAL shrimp. Table 4.3 summarizes all genera/species found in this study. It is shown that the most abundant genera (*Pseudoalteromonas*, *Psychrobacter*) and also the genera *Exiguobacterium* and *Planococcus* species were discovered via both PCR-DGGE analysis and isolation. The other genera were only discovered via plating and isolation (*Loktanella*, *Pseudomonas* and *Chryseobacterium*) or only via PCR-DGGE analysis (*Bacillus*, *Carnobacterium* and *Staphylococcus*).

Table 4.2. Identification of the excised PCR-DGGE bands shown in Fig 5 and 6 based on the 16S rRNA-V3 region from the microbiota on brown shrimp via similarity searches against the FASTA websearch. T_0= present at day one, T_1 present after 7 days of storage of shrimp, T_2= present after 13 days of storage of shrimp, ice= present during storage on ice, 7.5°C= present during storage at 7.5°C, L= microbiota recovered from swabs from modified Long and Hammer medium, P= microbiota recovered from swabs from plate count agar, D= microbiota recovered from the direct DNA extraction from the shrimp matrix, /= absent, n/a= not applicable.

Band	Closest relative in FASTA websearch	Similarity (%)	The best possible processing		Purchased	
			Unpeeled	Peeled	Unpeeled	Peeled
a	*Psychrobacter* sp.	89.8	T_0 ice (P & L)	T_1 ice (P)	/	/
b	*Planococcus donghaensis*	99.3	T_0 ice (P, L)	T_1 ice (P) T_2 ice & T_1 7.5°C (D)	T_0 and T_1 ice (L & D)	T_0 ice (D)
c	*Bacillus* sp.	99.3	T_1 at 7.5°C (L)	T_1 ice (L)	/	/
d	*Pseudoalteromonas translucida*	98.6	T_0 ice (L) & T_1 ice (L, D)	/	T_1 ice (L)	T_1 and T_2 ice (L)
e	*Pseudoalteromonas arctica* *Psa. fuliginea*	98.7-98.5	T_1 ice (L)	/	T_1 ice (L)	/
f	*Crangon crangon (brown shrimp)* [g]	97.6	n/a	n/a	n/a	n/a
g	*Exiguobacterium* sp.	89.1	T0, T1, T2 ice, T1 7.5°C (D)	T1, T2 ice, T1 7.5°C (D)	T0 ice (D)	T0 ice (D)
h	*Uncultured Verrucomicrobia bacterium (EU350866)*	77.6	T0 ice (L), T1 ice (P)	/	T0 ice (L), T1 & T1 ice (D)	T0 ice (D)
i	*Staphylococcus* sp.	89.7	/	/	T0 ice (D)	/
j	*Carnobacterium funditum* *C. divergens*	100 - 99.6	T0 ice (P)	T1 ice (P), T1 7.5°C & T2 ice (D)	T1 ice (D)	/
k	*Psychrobacter* sp.	94.5	T1 ice (P, D), T1 7.5°C (P, D), T2 ice (D)	T1 7.5°C (P, D), T2 ice (P, D)	T1 ice (D)	T1 ice (D)

[g] An artefact from the direct DNA extraction.

IV. Discussion

Shrimp are rich in NPN compounds that are easily metabolised by microorganisms (Liston, 1980). Microbiological growth and activity are the main cause of spoilage and contribute to the off-flavour and olfactory changes associated with spoiled seafood. The APC results show that brown IDEAL shrimp without preservatives caught and processed under the best possible conditions stored on ice have a microbiological shelf life of about two weeks before their APC exceeds 7 $\log_{10}$ cfu/g. The absence of *Enterobacteriaceae* indicates hygienic processing under these conditions. LAB, which dominate the microbiota of MAP stored seafood (Tryfinopoulou et al. 2002), were practically absent on the shrimp in this study, possibly because of the other storage conditions, namely aerobic (iced) storage. INDUSTRIAL shrimp without preservatives stored on ice have a microbiological shelf life of about one week. The shorter microbiological shelf life of INDUSTRIAL shrimp shows that many factors (e.g., icing and limiting cross-contamination with raw material) could be manipulated to extend the shelf life of fresh shrimp. 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 cooking process severely decreased the bacterial contamination on the shrimp. Niamhuy et al. (2007) observed a 2 log reduction on white shrimp (*Penaeus indicus*) when a boiling time of 1 minute was used and a 4 log reduction when cooked for 3 to 4 minutes. Despite cooking during a similar duration, the microbiological reduction in this study for *Crangon crangon* was much lower than for *Penaeus indicus*. This can be explained by different reasons: (1) Microbial analysis was performed one day after cooking instead of immediately. (2) This can be due to cooling of the shrimp to the air (IDEAL shrimp) or with seawater (INDUSTRIAL shrimp). Using seawater to expedite the cooling process can be an important source of recontamination (Van Spreekens and de Man 1970). The IDEAL shrimp were first air-cooled then placed on ice, which quickly reduced their temperature to 4°C. However, less probable, some of the microorganisms remaining after the cooking process could possibly be able to survive the high temperatures and were able to grow out during ice storage. Cooking at a higher temperature or during a longer time might resolve this, but nothing is known about the heat resistance of these microorganisms. A change in the cooking and cooling process can also affect the taste and texture of the shrimp (Niamnuy et al., 2007). The cooking process itself may also influence the rate of bacterial outgrowth on the shrimp flesh during further storage, since the shell and flesh will be damaged (Dykes et al. 2003; Niamnuy et al. 2007).

In this study, both manual and machinal peeling of the shrimp resulted in equal reductions in APC. This indicates that manual peeling of the shrimp (as sterile as possible) was not more effective in reducing the APC. These results agree with Schrubring and Meyer (1999) who studied the difference in APC between hand-peeled and machine-peeled shrimp. As Dykes et al. (2003) discuss, these results can be explained by the fact that the bacterial contamination is not only present on the shell, but also in the crevasses and channels between the shell segments, which makes it impossible to peel the shrimp without contaminating the meat. Some microorganisms were found exclusively on INDUSTRIAL peeled shrimp as shown by PCR-DGGE band i (*Staphylococcus* sp.), which may indicate process contamination.

This study reinforces the finding that the storage temperature of seafood has an important effect on shelf life and microbiological growth. Immediately placing shrimp in a large amount of ice is recommended, as this can double the shelf life of shrimp without preservatives compared to storage at 7.5°C. The average refrigerator temperature of 7.5°C (± 0.5°C) was used in accordance with Marklinder et al. (2004) and the results from a fridge temperature study performed by consumers (WIV 2006). However, this temperature was considered worst case practice in this study. Several studies have indicated the importance of storage temperature on the enumeration of microorganisms as well as on sensory spoilage (Huidobro et al. 2002). In particular, TVC analysis showed a steep increase in H_2S producers under refrigerated storage conditions. One of the H_2S producers isolated from iron agar was identified as an *Exiguobacterium* sp. In this study, all the microorganisms identified on shrimp during storage were marine food-related microorganisms. Most of them have been isolated before from food or seafood. Some of the genera found are even known to contribute to seafood spoilage, such as *Pseudomonas* sp., *Bacillus* sp. and *Carnobacterium* sp. Members of the genus *Pseudomonas* tend to grow very rapidly. They often outgrow species of other genera (Moore et al. 2006) and have often been observed to dominate the microbiota of seafood stored aerobically under chilled conditions (Tryfinopoulou et al. 2002). This leads them to be used as a spoilage indicator (Olafsdottir et al. 2006). *Staphylococcus* species are often associated with post-harvest and processing procedures. The isolates found in this study were not closely related to *Staphylococcus aureus*, a known threat to public health. *Carnobacterium* is a typical genus present in MAP-stored food such as brined shrimp (Dalgaard et al. 2003). It has been assumed that this microorganism's presence is a result of contamination after the cooking process (e.g., cooling, peeling, or packaging), but in this study a species of the genus was found based on identification of a PCR-DGGE band (k) on

unpeeled IDEAL shrimp as well. This species was closest related to *Carnobacterium funditum* and *Carnobacterium divergens* with a similarity of 100 to 99.6%. This indicates that the presence of this genus may not be the result of contamination post-cooking but may possibly originate from the intestinal content and survive the cooking process. *Carnobacterium divergens* has been reported before in cooked and brined shrimp (Dalgaard et al. 2003). Strains belonging to this genus have previously been isolated from tropical cooked and peeled shrimp (Jaffrés et al. 2009). The other genera found in this study have been correlated to seafood before, but to date those have not been described as SSOs. Some studies indicate, however, that some of these organisms may have spoilage potential. Species of the genus *Chryseobacterium*, formerly known as *Sejongia* (Kampfer et al. 2009), have been isolated from fish (Austin and Austin 1999) and some of the strains may contribute to sensory spoilage of fish (Engelbrecht et al. 1996). Many *Planococcus* species, formerly known as *Micrococcus*, were assigned to this genus the last decade and have been isolated from fish and other cold or frozen food (Hao and Komagata 1985). Alvares et al. (1982) described that proteolytic *Planococcus* species may contribute to shrimp spoilage. However, *Planococcus donghaensis,* the closest phylogenetic neighbour of the strains isolated in the present study, is not able to produce hydrogen sulphide and other spoilage characteristics are not known (Choi et al. 2007). To date, no *Exiguobacterium* species have been isolated from fish, therefore nothing is known about their possible role in shelf life. In this study, *Exiguobacterium* sp. seemed to be an hydrogen sulphide producer on iron agar.

Pseudoalteromonas and *Psychrobacter* dominate the microbiota of brown shrimp without preservatives regardless of differences in area and season of catch, early handling, processing procedures, and storage conditions. Both genera are described quite recently. The genus *Pseudoalteromonas* was described in 1995, and contains several species previously described as *Alteromonas*, *Shewanella* and *Moritella* (Gauthier et al. 1995). Likewise, the genus *Psychrobacter* was first described in 1986 (Juni and Heym 1986) and most species have only been identified during the last decade. As also shown in chapter 3, both genera are often observed to be the most abundant microorganisms on several fish species and other seafood during storage on ice or on fish in general (Reynisson et al. 2009; Romero et al. 2002; Wilson et al. 2008). Both genera have been associated with the gut microorganisms of lobsters, prawns and other seafood (Fjellheim et al. 2007; Meziti et al. 2010; Oxley et al. 2002). Both genera were also shown to cause black spots (melanosis), a problem relevant to the storage of raw shrimp (Miwa et al. 2008).

Table 4.3. Summarizing table with all the identified species or genera found on brown shrimp during storage, with indication of the sample from which they were found, the time points during storage, the processing procedures, the storage temperature and via which technique they were recovered and identified. The last column shows on which growth media the genus or species was isolated/detected. T_0= present at day 1, T_1= present after 7 days of storage, T_2= present after 13 days of storage. S= microbiota recovered from plate swabs, D= microbiota recovered from the direct DNA extraction from the shrimp matrix. LH= modified Long and Hammer medium, PCA= plate count agar, IA= Lyngby iron agar, MA= marine agar, CFC= *Pseudomonas* Cetrimide Fucidine Cephaloridine agar

Genus/species	Shrimp sample	Time point	Processing	Storage conditions	Detected/identified via	Growth media
Pseudoalteromonas						
Psa. nigrifaciens	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	Rep and *gyr*B	
Psa. elyakovii	Ideal processing	T_1	Both unpeeled and peeled	At both temperatures	Rep and *gyr*B	
Psa. paragorgicola	Ideal processing	T_1, T_2	Both unpeeled and peeled	On ice	Rep and *gyr*B	All media except PCA
Psa. translucida	Ideal processing	T_0, T_1	Unpeeled	On ice	PCR-DGGE (S,D) 16S-V3	
	Purchased	T_1, T_2	Both unpeeled and peeled	On ice	PCR-DGGE (S) 16S-V3	
Psa. arctica / Psa. fuliginea	Ideal processing	T_1	Unpeeled	On ice	PCR-DGGE (S) 16S-V3	
	Purchased	T_1	Peeled	On ice	PCR-DGGE (S) 16S-V3	
Pseudomonas						
Pseudomonas sp.	Ideal processing	T_1	Peeled	On ice	Rep and 16S	CFC
Psychrobacter						
Psychrobacter sp.	Ideal processing	T_1, T_2	Both unpeeled and peeled	On ice	PCR-DGGE (S) 16S-V3	PCA, LH
	Ideal processing	T_0	Unpeeled	On ice	Rep and *gyr*B	PCA, LH
Psb. cibarius	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	Rep and *gyr*B	All media
Psb. immobilis	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	Rep and *gyr*B	PCA
Psb. okhotskensis	Ideal processing	T_1	Peeled	At both temperatures	Rep and *gyr*B	PCA
Psb. marincola	Ideal processing	T_0	Unpeeled	On ice	Rep and *gyr*B	PCA
Staphylococcus						
Staphylococcus sp.	Purchased	T_0	Unpeeled	On ice	PCR-DGGE (D) 16S-V3	

4 **Table 4.3.** Continue

Genus/species	Shrimp sample	Time point	Processing	Storage conditions	Detected/identified via	Growth media
Bacillus						
Bacillus sp.	Ideal processing	T_1	Both unpeeled and peeled	At both temperatures	PCR-DGGE (S) 16S-V3	LH
Carnobacterium						
C. funditum/ C. divergens	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	PCR-DGGE (S, D) 16S-V3	PCA
Chryseobacterium						
Chryseobacterium sp.	Ideal processing	T_1	Peeled	At 7.5°C	Rep and 16S	PCA
Exiguobacterium						
Exiguobacterium sp.	Ideal processing	T_1	Unpeeled	At 7.5°C	Rep and 16S	IA
	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	PCR-DGGE (D) 16S-V3	
	Purchased	T_0	Both unpeeled and peeled	On ice	PCR-DGGE(D) 16S-V3	
Loktanella						
Loktanella sp.	Ideal processing	T_0	Unpeeled	On ice	Rep and 16S	MA
Planococcus						
Planococcus sp.	Ideal processing	T_0	Unpeeled	On ice	Rep and 16S	MA
	Ideal processing	T_1	Peeled	At 7.5°C	Rep and 16S	MA
Planococcus donghaensis	Ideal processing	All time points	Both unpeeled and peeled	At both temperatures	PCR-DGGE (S,D) 16S-V3	PCA, LH
	Purchased	T_0 and T_1	Both unpeeled and peeled	On ice	PCR-DGGE (S,D) 16S-V3	LH

Several species of the genus *Pseudoalteromonas* have many useful properties, such as antimicrobiological properties, the ability to break down PCBs (Michaud et al. 2007) and the degradation of paralytic shellfish toxins (Donovan et al. 2009). Little is known about the spoilage potential of those microorganisms, but some species such as *Pseudoalteromonas citrea* have proteolytic activities (Iijima et al. 2009) and are able to hydrolyse fish proteins (Belchior and Vacca 2006). Ivanova et al. (1998) revealed that *Pseudoalteromonas fuliginea*, which might also be found in this study (PCR-DGGE band e (Fig. 4.6 and Table 4.2)), is a heterotypic synonym of *Pseudoalteromonas citrea*. Some *Pseudoalteromonas* species form biofilms, which may affect processing of seafood and cleaning of the appliances used in processing.

Species of the genus *Psychrobacter* are members of the spoilage microbiota of chilled proteinaceous foods stored in air (Bowman 2006). *Psychrobacter* species have been isolated from several seafood products such as cooked and peeled or frozen tropical and nordic shrimp in MAP packaging (Jaffrés et al. 2009; Mejlholm et al. 2005; Tsironi et al. 2009), salt-cured cod (Bjorkevoll et al. 2003) and shellfish (Prapaiwong et al. 2009). Their importance in spoilage is considered rather low since they seemed to be unable to compete with common spoilage microorganisms (Rodriguez-Calleja et al. 2005); however, their spoilage potential has not been extensively studied. *Psychrobacter immobilis* is able to produce only weak slightly fishy, musty (Mejlholm et al. 2005; Prapaiwong et al. 2009) off-flavours, and is able to hydrolyse lipids (Gennari et al. 1992; Yumoto et al. 2003).

It was shown that the use of the 16S rRNA gene for identification of shrimp isolates resulted in species complexes within the genera *Psychrobacter* and *Pseudoalteromonas* since similarity searches against type strains in the *Eztaxon* database resulted in plural species names with more than 98.7% similarity and often with either null, one or two base pairs difference. Identification based on the *gyr*B gene could better discriminate between species (La Duc et al., 2004).

When a 16S rRNA gene based phylogenetic tree was made, type strains of the species *Pseudoalteromonas distincta* (AF082564), *Pseudoalteromonas paragorgicola* (AY040229) and *Pseudoalteromonas elyakovii* (AF082562) were not distanced from each other. By using *gyr*B, the sequence differences between those type strains was larger, and identification of some of the isolates could be achieved. Not many *gyr*B sequences of type strains had been deposited in a public database, whereas *gyr*B sequences of several type strains were deposited

in the EMBL nucleotide database. This allowed the use of a phylogenetic tree with inclusion of these type strains for isolate identification. Most isolates were identified as *Pseudoalteromonas nigrifaciens* (similarity > 99.1%).

Psychrobacter identification showed similar problems. Based on *gyr*B sequences, those isolates were identified as either *Psychrobacter cibarius* or *Psychrobacter immobilis* (similarity between 96.3 and 99.8%). A phylogenetic tree (Fig 4.3) indicated some sequence differences between all isolates; clear allocation to either one of the two species was not possible. This could mean that the tree also indicates the possible presence of some new species within this genus (isolate G30, the cluster G12, 1G192, 1G201 and the cluster G71, G56, G67, G34, G70).

PCR-DGGE analysis using the 16S-V3 region has often been used in seafood research to observe population shifts and identify the microbiota present. In this study, PCR-DGGE was used to observe population shifts during storage and among different storage conditions. Only few differences between unpeeled and peeled shrimp were observed, and the differences were mostly not very large. This can be explained by the very high incidence of contaminating the meat while peeling the shrimp either manually or with a machine (Dykes et al. 2003). Differences between culture-dependent and culture-independent techniques were also observed using PCR-DGGE analysis. Food science research relies on many factors, including the choice of growth media or DNA extraction methods. Some differences were observed between both culture-dependent and independent detection of microbiota such as in the dominance of *Psychrobacter* and *Pseudoalteromonas* (Fig. 4.5 and 4.6). When using the culture-independent method, the genera were still found but the intensity of the bands was much weaker, especially for the band corresponding with *Psychrobacter*. This may indicate that on growth media *Psychrobacter* spp. and *Pseudoalteromonas* spp. have a slight advantage and possibly outgrow some other microorganisms. The difference in band pattern can also arise from the media used (Table 4.3). For example, *Exiguobacterium* sp. (Fig 4.6; band h) was isolated from Lyngby iron agar (IA) and could only be identified from all culture-independent PCR-DGGE patterns since plate swabs from IA were not included in the PCR-DGGE analysis. The inability of microorganisms to grow on PCA or LH might explain the differences in PCR-DGGE patterns (Table 4.3). As previously observed in chapter 3, some *Psychrobacter* spp. were unable to grow on the LH growth medium. In contrast, *Pseudoalteromonas* spp. were only detected on LH. The PCR-DGGE technique itself also has

some potential pitfalls, such as a detection limit of 10^3 cfu/g, which also depends on the species composition of the total bacterial community (Muyzer et al. 1993). The abundance of some species may mask the presence of the other microorganisms, even when they are present in higher numbers than 10^3 cfu/g. Sequence heterogeneity of the 16S gene might cause the diversity to be overestimated (de Araujo and Schneider 2008). Identification of the PCR-DGGE bands is limited to genus level due to the very small fragment sequenced. Potential co-migration of different species as one band or as bands with the same migration pattern might compromise the identification and presence of species in samples based on comparison of bands. This must therefore be interpreted with caution.

In conclusion, *Pseudoalteromonas* and *Psychrobacter* are the dominant microbiota of brown shrimp during storage on ice and at 7.5°C. Both genera were present on IDEAL shrimp processed under the best possible circumstances as well as from INDUSTRIAL samples. Culture-dependent techniques more clearly revealed the presence of those genera. When the culture-independent technique PCR-DGGE was used, both genera were still present, but their presence in every sample diminished especially for *Psychrobacter* spp., while some other bands in the PCR-DGGE pattern became more pronounced. Therefore, when studying the microbiota on seafood one needs to account for the inability of some techniques to give a complete overview or indicate which microbiota are present.

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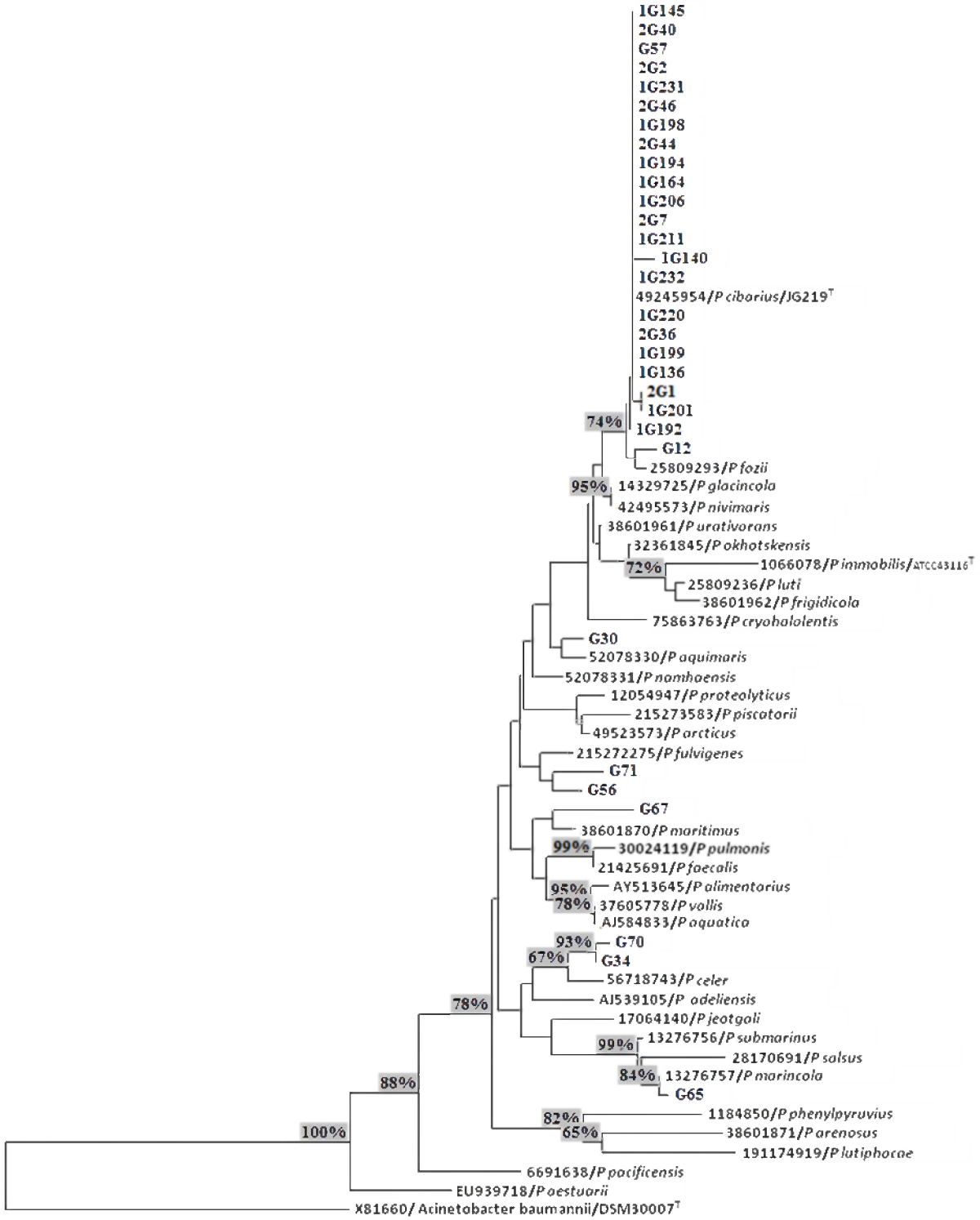
Supplementary table 4.1. Tentative identification of the isolates from brown shrimp belonging to the genus *Psychrobacter* via the nearest phylogenetic neighbour based on the full 16S rRNA gene (1500bp) and the *gyr*B gene (1500bp) with indication of the percentage of similarity.

Isolate names	**16S EZTAXON (accession number)**	**similarity (%)**	*Gyr*B **Fasta (accession number)**	**similarity (%)**
G12	*Psb. fozii* (AJ430827)	99.1%	*Psb. cibarius* (FR668579)	96.2%
G30	*Psb. maritimus* (AJ609272)	97.4%	*Psb. maritimus* (FR668575)	87.6%
G34	*Psb. celer* (AY842259)	99.1%	*Psb. marincola* (DQ143914)	85.8%
G56	*Psb. namhaensis* (AY722805)	97.9%	*Psb. maritimus* (FR668575)	85.3%
G57	*Psb. fozii* (AJ430827)	99.6%	*Psb. immobilis* (DQ143927)	98.5%
G65	*Psb. marincola* (AJ309941)	100%	*Psb. marincola* (DQ143919)	97.9%
G67	*Psb. maritimus*(AJ609272)	98.2%	*Psb. submarinus* (DQ143923) *Psb. marincola* (DQ143919)	85.0%
G70	*Psb. celer* (AY842259)	98.9%	*Psb. marincola* (DQ143919)	85.9%
G71	*Psb. nivimaris* (AJ313425)	94.9%	*Psb. pulmonis* (AB458223)	85.1%
1G136	*Psb. cibarius* (AY639871)	98.9%	*Psb. immobilis* (FR668577)	98.1%
1G140	*Psb. cibarius* (AY639871)	97.7%	*Psb. cibarius* (FR668579)	97.3%
1G145	*Psb. cibarius* (AY639871)	98.1%	*Psb. cibarius* (FR668579)	98.1%
1G164	*Psb. cibarius* (AY639871)	100%	*Psb. cibarius* (FR668579)	97.1%
1G192	*Psb. cibarius* (AY639871)	100%	*Psb. immobilis* (DQ143927)	96.3%%
1G194	*Psb. cibarius* (AY639871)	100%	*Psb. okhotskensis* (DQ143920)	91.1%
1G198	*Psb. cibarius* (AY639871)	100%	*Psb. cibarius* (FR668579)	98.7%
1G199	*Psb. cibarius* (AY639871)	98.3%	*Psb. immobilis* (DQ143927)	96.8%
1G201	*Psb. cibarius* (AY639871)	100%	*Psb. cibarius* (FR668579)	96.9%
1G206	*Psb. cibarius* (AY639871)	100%	*Psb. immobilis* (DQ143927)	96.5%
1G211	*Psb. cibarius* (AY639871)	97.9%	*Psb. immobilis* (DQ143927)	96.5%
1G220	*Psb. cibarius* (AY639871)	98.3%	*Psb. cibarius* (FR668579)	97.8%
1G231	*Psb. cibarius* (AY639871)	100%	*Psb. cibarius* (FR668579)	97.0%
1G232	*Psb. cibarius* (AY639871)	97.6%	*Psb. cibarius* (FR668579)	99.8%
2G1	*Psb. cibarius* (AY639871)	99.2%	*Psb. cibarius* (FR668579)	96.8%
2G2	*Psb. cibarius* (AY639871)	100%	*Psb. immobilis* (DQ143927)	96.8%
2G36	*Psb. cibarius* (AY639871)	98.4%	*Psb. cibarius* (FR668579)	97.5%
2G40	*Psb. cibarius* (AY639871)	100%	*Psb. cibarius* (FR668579)	99.1%
2G44	*Psb. cibarius* (AY639871)	98.3%	*Psb. immobilis* (FR668577)	96.8%
2G46	*Psb. cibarius* (AY639871)	98.8%	*Psb. cibarius* (FR668579)	96.3%

Supplementary table 4.2. Tentative identification of the isolates from brown shrimp belonging to the genus *Pseudoalteromonas* via the nearest phylogenetic neighbour based on the full 16S rRNA gene (1500bp) and the *gyr*B gene (1500bp) with indication of the percentage of similarity. /: not performed.

Isolate names	**16S EZTAXON (accession number)**	**similarity (%)**	*Gyr*B **Fasta (accession number)**	**similarity (%)**
G41	/	/	*Psa. arctica* (FR668562)	96.4%
G43	*Psa. nigrifaciens (*X82146) *Psa. elyakovii* (AF082562) *Psa. undina* (X82140)	98.5%	*Psa. nigrifaciens* (FR668569)	99.1%
1G146	*Psa. elyakovii* (AF082562)	98.7%	*Psa. nigrifaciens* (FR668569)	99.4%
1G148	*Psa. elyakovii* (AF082562) / *Psa. espejiana* (X82143)	98.5%	*Psa. nigrifaciens* (FR668569)	99.6%
1G161	*Psa. nigrifaciens (*X82146)	97.5%	*Psa. elyakovii* (FR668565)	98.7%
1G209	*Psa. elyakovii* (AF082562) *Psa. nigrifaciens (*X82146)	98.8%	*Psa. nigrifaciens* (FR668569)	99.6%
1G212	*Psa. arctica* (DQ787199) *Alteromonas fuliginea* (AF529062) *Psa. elyakovii* (AF082562 *Psa. nigrifaciens (*X82146)	98.1%	*Psa. nigrifaciens* (FR668569)	99.6%
1G215	*Psa. nigrifaciens (*X82146)	98.0%	*Psa. nigrifaciens* (FR668569)	98.8%
1G242	*Psa. nigrifaciens (*X82146) *Psa. elyakovii* (AF082562)	99.3%	*Psa. nigrifaciens* (FR668569)	99.1%
1G244	*Psa. nigrifaciens (*X82146)	98.0%	*Psa. nigrifaciens* (FR668569)	99.6%
1G272	*Psa. nigrifaciens (*X82146) *Psa. elyakovii* (AF082562) *Psa. haloplanktis* (X67024)	100%	*Psa. elyakovii* (FR668565)	98.7%
2G19	*Psa. elyakovii* (AF082562)	98.8%	*Psa. nigrifaciens* (FR668569)	99.4%
2G20	*Psa. elyakovii* (AF082562)	98.4%	*Psa. nigrifaciens* (FR668569)	99.7%
2G22	*Psa. elyakovii* (AF082562)	98.7%	*Psa. nigrifaciens* (FR668569)	99.7%
2G68	*Psa. translucida* (AY040230)	99.6%	*Psa. paragorgicola*(FR668570) / *Psa. elyakovii* (FR668565)	98.9%



Suppl. Fig 4.1. Phylogenetic tree of the genus *Psychrobacter* based on the full 16S rRNA gene sequence (1500bp). The isolates found in this study were hereby compared to all sequences generated from the typestrains of the species within this genus. The tree was constructed with the neighbour-joining method. Genetic distances were calculated by the Jukes & Cantor model. The scale shows the genetic distance of 0.02. The percentages on the nods give the bootstrap values (on 1000 bootstrapped trees). Only values above 65% are shown. *Acinetobacter baumannii* was used as outgroup.

CHAPTER 5

VOLATILE COMPOUNDS ASSOCIATED WITH *PSYCHROBACTER* SPP. AND *PSEUDOALTEROMONAS* SPP., THE DOMINANT MICROBIOTA OF BROWN SHRIMP (*CRANGON CRANGON*) DURING AEROBIC STORAGE

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Chapter 5. Volatile compounds associated with *Psychrobacter* spp. and *Pseudoalteromonas* spp., the dominant microbiota of brown shrimp (*Crangon crangon*) during aerobic storage

Abstract

Psychrobacter and *Pseudoalteromonas* species dominate the microbiota of cooked brown shrimp (*Crangon crangon*). Therefore, the spoilage potential of several *Psychrobacter* and *Pseudoalteromonas* species (*Psychrobacter cibarius*, *Psychrobacter maritimus*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas paragorgicola* and *Pseudoalteromonas nigrifaciens*) was determined and quantified based on the presence of VOCs. Additionally, API ZYM analyses determined the species' enzymatic capacity to contribute to spoilage by degrading lipids, amino acids and proteins. The bacterial species used in this study were isolated from cooked brown shrimp during storage (spoilage) under different storage and processing (peeled, unpeeled) conditions and were selected for analysis of their spoilage potential based on their difference in the $(GTG)_5$-rep profile, 16S rRNA and *gyr*B sequences and API ZYM profile. The isolates were inoculated as pure cultures on heat-sterilised shrimp. The inoculated samples were stored at 4°C and the production of VOCs by the pure strains on the shrimp matrix was identified via gas chromatography coupled to mass spectrometry (GC-MS). VOC production was quantified daily by selected ion flow tube mass spectrometry (SIFT-MS) until the bacterial count exceeded 10^8 - 10^9 cfu/g. Based on the API ZYM results, *Pseudoalteromonas* as well as *Psychrobacter* species might enhance spoilage by breaking down lipids and hydrolysing amino acids and proteins. The sensory profile of *Psychrobacter* species revealed very low potential of the production of VOCs. *Pseudoalteromonas* species, especially *Pseudoalteromonas elyakovii* and *Pseudoalteromonas nigrifaciens*, produced significant amounts of volatile compounds such as sulphides, acetone, ammonia, and ethanol, which are all involved in seafood spoilage, and might be responsible for the off-odours produced during spoilage of brown shrimp.

I. Introduction

As described in chapter 2, seafood spoilage is a complex phenomenon involving various biochemical and microbiological factors. Microbiological growth and activity is by far the most common cause of spoilage and contributes to the textural, off-flavour, and olfactory changes associated with spoiled seafood. Brown shrimp are prone to rapid microbiological spoilage due to the ideal intrinsic conditions: a nearly neutral pH, a high water activity ($a_w = \pm 0.99$) and high content of low molecular weight compounds, which are easily metabolised by microorganisms (Liston 1980). Bacterial degradation of soluble, low molecular weight components results in the formation of volatile metabolites such as alcohols, ketones, sulphur compounds, amines (TMA, DMA), esters, aldehydes, and organic acids (Gram and Dalgaard 2002; Gram et al. 2002). Some of these metabolites are responsible for the unpleasant and offensive off-odours and off-flavours that lead to sensory rejection and shorten the shelf life of seafood (Gillespie and Macrae 1975; Herbert et al. 1971; Shewan and Murray 1979). These VOCs are metabolised by only a fraction of the microbiota present on the seafood during storage, generally referred to as SSOs (Dalgaard 1995).

Chapter 4 revealed that the genera *Psychrobacter* and *Pseudoalteromonas* dominate the microbiota of brown shrimp without preservatives despite differences in area and season of catch, early handling and processing procedures, or storage conditions. Their abundant presence has also been observed on other fish and fishery products during storage on ice or on fish in general (see chapter 3). However, literature contains little data on the spoilage potential of these organisms. Several species of the genus *Pseudoalteromonas* have been used for their antimicrobiological properties, their ability to break down PCBs (Michaud et al. 2007), and their ability to degrade paralytic shellfish toxins (Donovan et al. 2009). Some, such as *Pseudoalteromonas citrea*, have proteolytic activities (Iijima et al. 2009) and are able to hydrolyse fish proteins (Belchior and Vacca 2006). They are known to form biofilms, which may be important in seafood processing and cleaning of the appliances.

Species of the genus *Psychrobacter* belong to the group of spoilage microbiota found on chilled proteinaceous foods stored in air (Bowman 2006) and have been isolated from several seafood products (see chapter 3). Only one species within the genus, *Psychrobacter immobilis* has been reported to produce slightly fishy, musty off-flavours (Mejlholm et al. 2005; Prapaiwong et al. 2009) and has the capacity to hydrolyse lipids (Gennari et al. 1992; Yumoto et al. 2003).

The aim of this study was to investigate the spoilage potential of several *Psychrobacter* and *Pseudoalteromonas* spp. isolates that had been previously isolated from cooked peeled and unpeeled brown shrimp without preservatives stored under aerobic conditions. We studied the spoilage potential of these isolates by studying the sensory profile of an inoculated pure culture on sterile shrimp as detected by gas chromatography coupled to mass spectrometry (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. Material and methods

2.1. Selection of bacterial strains based on API ZYM characterisation

From a collection of *Psychrobacter* and *Pseudoalteromonas* isolates from brown shrimp (*Crangon crangon*) described in chapter 4, a selection of isolates was made which represented $(GTG)_5$-rep PCR fingerprint-clusters and different species based on partial 16S rRNA gene sequencing. In total, 17 isolates were selected for API ZYM tests (Biomerieux): 6 isolates from the genus *Pseudoalteromonas* and 11 from the genus *Psychrobacter*. This test was used to determine their enzymatic activities for the following reasons: (1) to indicate the probable (biochemical) spoilage activity and (2) to further clarify strain differences in addition to $(GTG)_5$-rep fingerprints and sequence identification. In total, we performed 19 enzymatic tests: 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-glucuronidase; α-glucosidase; β-glucosidase; N-acetyl-β-glucosaminidase; α-mannosidase; and α-fucosidase. The isolates were cultured on modified Long and Hammer medium (Van Spreekens 1974) at 21°C for 5 days since they are unable to grow on regular plate agar (see chapter 3). Further analysis was performed according to the manufacturers' guidelines with the exception of incubating the strips at 21°C for 20 h. The API ZYM results were used to select the isolates for further study of the volatile compounds (see 2.4.). Isolates with the same $(GTG)_5$–rep PCR fingerprint, same sequence identification, and same API ZYM results were considered to be identical. The selected isolates were identified to species level by *gyr*B gene sequencing as described in chapter 3.

2.2. Sample inoculation and storage

For each of the selected isolates, 1 kg fresh shrimp without preservatives in a 5L Duran bottle was heat-treated at 121°C for 10 min in a pressure cooker. The bottles were then immediately

stored at 4°C. After rapid cooling to 4°C in the fridge on ice, the remaining moisture was poured out of the bottles in a sterile manner and the sterile shrimp were inoculated with 10^2 to 10^4 cfu/g of each isolate. One bottle of sterile shrimp was not inoculated and was used as a control. All bottles were stored at 4 C until the end of the experiment (T_1-T_9). Samples were aseptically taken daily starting the day after inoculation (T_1) for bacterial enumeration and to identify (GC-MS) and quantify (SIFT-MS) the volatiles (see further).

2.3. Total counts of inoculated samples, pH and lactic acid production

The growth of the bacterial isolates on the samples and pH of every sample was measured daily. For the microbiological analysis, 10 g of shrimp 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 Long and Hammer medium (Van Spreekens 1974) 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 measured with a pH meter (Mettler Toledo) by mincing 5 to 10 g of shrimp sample. An RI-HPLC analysis according to Dang et al. (2009) was performed to measure the production of lactic acid within 4 weeks after sampling in samples stored at -20°C.

2.4. Analysis of spoilage related VOCs

2.4.1. Identification of VOCs by GC-MS

Every other day starting at T_1, shrimp from each inoculated and control sample were aseptically prepared for HS-GC-MS in order to identify all volatile compounds produced by the isolates. This was done by placing 5.0 ± 0.1 g of each sample in a 20ml glass vial closed with a PTFE-faced silicone septum crimp cap (Agilent Technologies, Diegem, Belgium). During an incubation period of 30 min, samples were heated at 50 ± 0.5°C. One ml of the headspace of the vial was sampled with the headspace CTC PAL auto sampler (Agilent Technologies, Belgium) in the PTV injector (Agilent Technologies, Belgium) of the GC. Chromatographic separation was performed on a capillary DB-WAX column of 60m, 0.25mm ID, 0.25µm film thickness, in a 7890A GC system (Agilent Technologies, Diegem, Belgium) with a constant flow of 0.8 ml helium/min. The temperature programme was 5 min at 45 °C, ramp 7 °C/min to 220 °C, 10 min at 220°C and a 10 min post-run at 230 °C. An Agilent 5975 Series electron impact ionization mass spectrometer (Agilent Technologies, Diegem, Belgium) with 70 eV ionization energy operating in full scan mode with a mass range

between 33 and 330 was used for detection. Interface, source and quadruple temperatures were 270 °C, 230 °C and 150 °C, respectively. The MSD Chemstation software package was used for data processing. The compounds were identified based on the retention time as well as by spectral comparison using the NIST 05 library. In total, 17 compounds (Table 5.1) were 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, were considered to be natural compounds of the shrimp matrix, and were therefore not selected. Real-time quantification of the 17 compounds was performed using SIFT-MS.

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

Every day, starting with T_1, 50.0 ± 0.5g of shrimp from each inoculated and blank sample, stored in air at 4°C, was aseptically taken until a total viable count (TVC) of 10^8 cfu/g was exceeded (T_9). Each 50g shrimp sample was packed in a high barrier film bag (Euralpack, Schoten, Belgium) with 950.0 ± 5.0ml of inert N_2 gas using a Multivac A300/42 packaging unit (Hagenmüller, Wolfert-schwenden, Germany) for SIFT analysis as described in Noseda et al (2010). All bags were stored at 4°C for 1 h before starting SIFT analysis. The headspace was sampled through selected based on the HS-GC-MS results (Table 5.2) and a preliminary research with HS-GC-MS a septum on the sampling bag during 60 seconds with a flow of 77.3 Pa L s^{-1}. VOCs were introduced through the heated inlet into the flow tube, where reactions with precursor ions H_3O^+, NO^+ and O_2^+ resulted in ionized masses. These masses were monitored by a mass spectrometer located at the downstream area of the flow tube. Specific VOCs were targeted using the multiple ion monitoring mode (MIM). The reaction rate coefficients (K) and the branching ratios between the precursor ions and the target VOCs were used to quantify the VOCs. Table 5.1 shows the ionized masses used for quantification. Blank samples (three empty sample bags filled with nitrogen) were randomly analysed between other samples. For every compound, a limit of detection (LOD) was calculated based on the mean value of the 3 blank samples (x_{bl}) plus three times the standard deviation (SD_{bl}):

$$LOD = x_{bl} + (3*SD_{bl})$$

LOD value was subtracted from the analysed VOC concentration of the inoculated samples. The reported concentrations ([sample]) are the mean value of the samples (x_{sample}) (only those above the LOD value), subtracted by the mean value of the blank sample (x_{bl}):

$$[sample] = x_{sample} - x_{bl}.$$

Table 5.1. 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					
ethanol	H_3O^+	47	100	2.70E -09	$C_2H_7O^+$
	H_3O^+	65		2.70E -09	$C_2H_7O^+.H_2O$
	H_3O^+	85		2.70E -09	$C_2H_7O^+.(H_2O)_2$
1,2-butanediol	NO^+	89	100	3.90E -09	$C_5H_{11}O^+$
2-propanol	H_3O^+	43	80	2.70E -09	$C_3H_7^+$
isobutyl alcohol	H_3O^+	57	100	2.70E -09	$C_4H_9^+$
	NO^+	73	95	2.40E -09	$C_4H_9O^+$
Ketones					
2-pentanone	H_3O^+	87	100	3.90E -09	$C_5H_{11}O^+$
	H_3O^+	105		3.90E -09	$C_5H_{11}O^+.H_2O$
	NO^+	116	100	3.10E -09	$NO^+.C_5H_{11}O$
butanone	NO^+	102	100	2.80E -09	$NO^+.C_4H_8O$
acetone	H_3O^+	59	100	3.90E -09	$C_3H_7O^+$
	H_3O^+	77		3.90E -09	$(CH_3)_2COH^+.H_2O$
	NO^+	88	100	1.20E -09	$NO^+.C_3H_6O$
Sulphur compounds					
methyl mercaptan	H_3O^+	49	100	1.80E -09	$H_3O^+.CH_4S$
sulphur hydride	H_3O^+	35	100	1.60E -09	H_3S^+
	O_2^+	34	100	1.40E -09	H_2S^+
dimethyl disulphide	H_3O^+	95	100	2.60E -09	$(CH_3)_2S_2.H^+$
	NO^+	94	100	2.40E -09	$(CH_3)_2S_2^+$
dimethyl thioether	NO^+	62	100	2.20E -09	$(CH_3)_2S^+$
Amines					
trimethyl amine	H_3O^+	58	10	2.00E -09	$C_3H_8N^+$
	H_3O^+	60	90	2.00E -09	$(CH_3)_3N.H^+$
dimethyl amine	H_3O^+	46	100	2.10E -09	$(CH_3)_2NH.H^+$
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	H_3O^+	90	100	9.00E -10	$NO^+.CH_3COOH$
Other					
ammonia	H_3O^+	18	100	2.70E -09	NH_4^+
	O_2^+	17	100	2.40E -09	NH_3^+
ethylene oxide	H_3O^+	45	100	2.40E -09	$C_2H_5O^+$
	NO^+	74	100	1.00E -10	$C_2H_4O.NO^+$

Table 5.2. GC-MS results on sterilized shrimp inoculated with *Pseudoalteromonas* and *Psychrobacter* strains and stored in air on ice. Analyses were performed after 7 ice storage. The compounds marked with an asterisk showed an increase during storage and were incorporated in the SIFT-MS method for quantification.

Component	*Pseudoalteromonas* spp.	*Psychrobacter* spp.
1,2-butanediol*	X	
1,2-butanone	X	
1-methoxy-butane	X	
2,3-dimethyl-oxirane	X	X
2,3-dimethyl-oxirane cis	X	
2-butanone	X	X
2-ethoxy-propane	X	
2-formylhistamine	X	X
2-methyl-2-propanol		X
2-methylfuran	X	
2-methyl-propanol	X	
2-pentanone*	X	
3-methyl-butanal	X	
Acetaldehyde		X
Acetone*	X	X
Dimethyl disulphide*	X	
Ethylene oxide*	X	X
Isopropyl alcohol	X	X
Methanethiol	X	
Trimethylamine*	X	X

This

III. Results

3.1. API ZYM results

The API ZYM results of the 17 isolates revealed differences in enzymatic activity. None of the 17 isolates were able to degrade one of the 12 carbohydrates included in the API ZYM strip. In general, members of the genus *Psychrobacter* showed some lipolytic (esterase (C4) and esterase lipase (C8)) activity and were capable of hydrolysing amino acids (leucine arylamidase). Members of the genus *Pseudoalteromonas* had a similar potential to degrade small lipids and hydrolyse amino acids, but some isolates also showed proteolytic (trypsin and α-chymotrypsin) activity. The phenotypical characteristics combined with the genotypic differences of the $(GTG)_5$-rep profiling described in chapter 4, resulted in the selection of 8 isolates for further spoilage analysis, i.e., 4 *Pseudoalteromonas* isolates and 4 *Psychrobacter* isolates. Table 5.3 shows the tentative *gyr*B gene identification of these selected isolates and the different enzymatic activities. Enzymatic differences between genera, differences between species, and variability between species were observed. Most of the tested isolates from the

genus *Pseudoalteromonas* showed more enzymatic activities than those of the genus *Psychrobacter*. For example, all *Pseudoalteromonas* isolates were positive for trypsin, while all *Psychrobacter* isolates were negative. Between species, differences were observed for alkaline phosphatase. Most species from *Pseudoalteromonas* were considered positive, except for isolate 2G68, identified as *Pseudoalteromonas paragorgicola*. For *Psychrobacter*, most isolates showed no alkaline phosphatase activity, except for *Psychrobacter maritimus* (1G200). Some variability was observed between tested isolates of the same species. In particular, lipase activities differed within the species *Pseudoalteromonas elyakovii* (esterase and esterase lipase) and between the isolates identified as *Psychrobacter cibarius* (esterase). *Pseudoalteromonas elyakovii* and *Pseudoalteromonas nigrifaciens* also showed variation for α-chymotrypsin activity between isolates.

Table 5.3. *Gyr*B identification and enzymatic activities of the selected isolates from shrimp 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).

Strain N°	*gyr*B ID (% of similarity by FASTA search)	APIZYM results											
		Phosphatases		Lipases				Hydrolysis of amino acids / proteins					Carbohydrates
		1	2	3	4	5	6	7	8	9	10	11	12
Pseudoalteromonas													
1G272	*Psa. elyakovii* (98.7%)	+	-	-	-	-	-	+	-	-	+	-	-
1G161	*Psa. elyakovii* (98.7%)	+	-	-	+	+	-	+	-	-	+	+	-
2G68	*Psa. paragorgicola*[8] (98.9%)	-	-	-	-	-	-	+	-	-	+	-	-
1G215	*Psa. nigrifaciens* (98.8%)	+	-	-	-	+	-	+	-	-	+	+	-
Psychrobacter													
1G198	*Psb. cibarius* (98.7%)	-	-	-	+	+	-	+	-	-	-	-	-
2G40	*Psb. cibarius* (99.1%)	-	-	-	-	+	-	+	-	-	-	-	-
1G232	*Psb. cibarius* (99.8%)	-	-	-	+	+	-	+	-	-	-	-	-
1G200	*Psb. maritimus* (87.6%)	+	-	-	-	+	-	+	-	-	-	-	-

[8] Same similarity to typestrain of *Pseudoalteromonas elyakovii*, however due to small sequence differences with the isolates 1G272 and 1G161 considered to be *Pseudoalteromonas paragorgicola*.

3.2. Total counts and pH

Figure 5.1 shows the results of bacterial growth on the inoculated sterile shrimp. The counts of the inoculated bacteria were 10^2 to 10^4 cfu/g at T_0. At T_9, nearly every sample except for 2G68 (*Pseudoalteromonas paragorgicola*) and 1G200 (*Psychrobacter maritimus*) exceeded 10^9 cfu/g. The pH of the samples inoculated with *Pseudoalteromonas* increased slightly during the first 3 days (from pH 8.17-8.21 to 8.24-8.34) and then decreased to 7.9 at T_9. The sample inoculated with *Pseudoalteromonas paragorgicola* did not reach 10^9 cfu/g after 9 days, which was reflected in a higher pH (8.16). The pH of the samples inoculated with *Psychrobacter* isolates followed a similar pattern and decreased after a small increase the first 4-5 days after inoculation from 8.21-8.39 to 8.1-8.19. The decrease of the pH was not correlated to lactic acid production, as HPLC analysis did not reveal any significant lactic acid and other acid production.

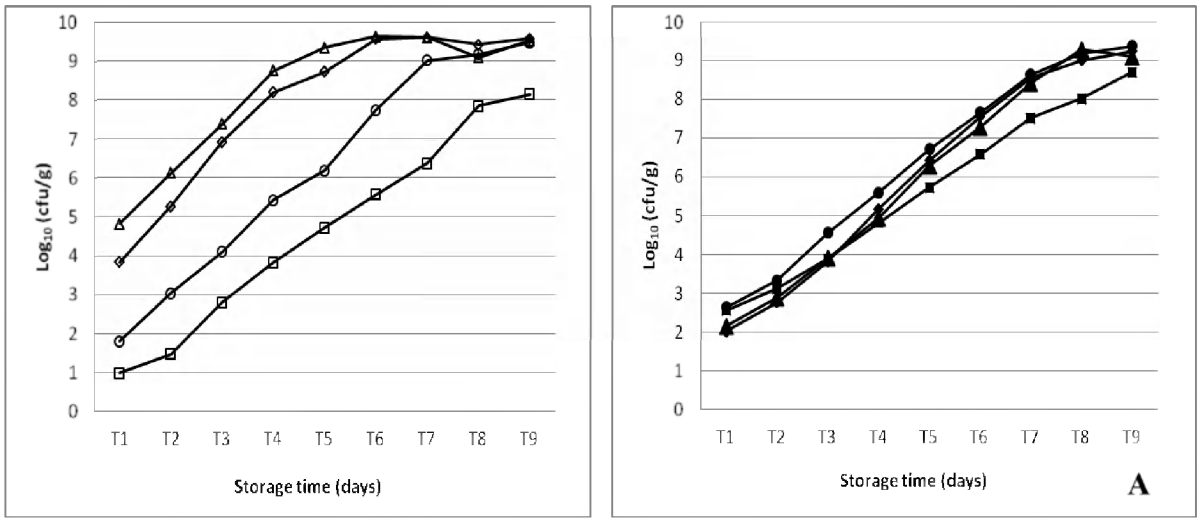

Figure 5.1. Growth of the bacterial isolates on chilled shrimp during storage at 4°C in air. The bacterial counts are given in $\log_{10}$ cfu/g. A: *Pseudoalteromonas* spp.: *Pseudoalteromonas nigrifaciens* (isolate 1G215 ◇), *Pseudoalteromonas elyakovii* (isolate 1G272 ○), *Pseudoalteromonas elyakovii* (isolate 1G161 △), *Pseudoalteromonas paragorgicola* (isolate 2G68 □). B: *Psychrobacter* spp.: *Psychrobacter cibarius* (isolate 2G40 ◆), *Psychrobacter cibarius* (isolate 1G198 ●), *Psychrobacter cibarius* (isolate 1G232 ▲), *Psychrobacter maritimus* (isolate 1G200 ■).

3.3. Volatile compounds

Table 5.2 shows the results of the GC-MS analysis with inoculated pure *Psychrobacter* and *Pseudoalteromonas* strains on sterilized shrimp stored on ice. The compounds marked with an asterisk clearly increased in concentration during storage and were selected for further analysis with the SIFT-MS. Table 5.1 shows the 17 VOCs selected by GC-MS analysis and by literature search for further analysis during storage of the inoculated shrimp samples.

Every day a quantitative SIFT-MS analysis for these 17 VOCs was performed on the inoculated samples and the blank sample during storage at 4°C.

In the samples inoculated with *Psychrobacter*, generally no significant production of VOCs was detected after 9 days of storage. However, two inoculated samples (one with *Psychrobacter cibarius* (isolate 1G232) and one with *Psychrobacter maritimus*) showed a higher concentration of TMA than all the other samples. The concentration of TMA was not clearly (linear) correlated with the total viable count, but did show some concordance with pH fluctuations as TMA and pH are always correlated with each other (Fig. 5.2).

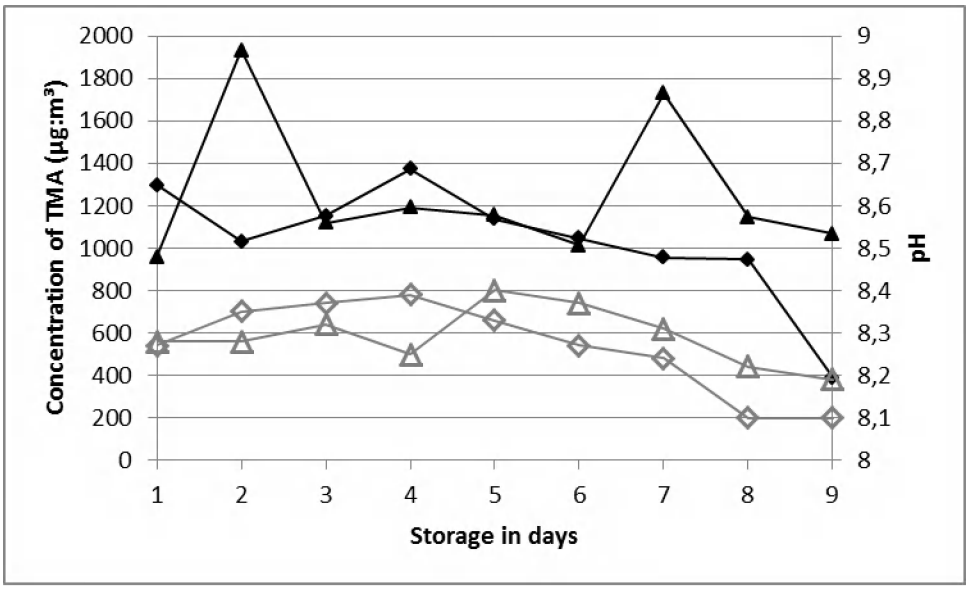

Fig. 5.2. pH and production of trimethylamine (TMA) (olfactory threshold by Devos et al., 1990: 5.89µg/m³) by *Psychrobacter cibarius* isolate 1G232 (◇ (pH) ◆ (TMA)) and *Psychrobacter maritimus* isolate 1G200 (△(pH) ▲ (TMA)), both in function of the storage time in days.

Several VOCs were detected and quantified in the samples inoculated with *Pseudoalteromonas* isolates. The most important compounds detected were 1,2-butanediol, 2-propanol, 2-pentanone, butanone, acetone, methyl mercaptan, sulphur hydride, dimethyl disulphide, ethyl acetate, acetic acid and ammonia. Sulphur compounds (i.e., sulphur hydride, methyl mercaptan and dimethyl disulphide (DMDS)) and acetone were produced by all isolates, but only for some isolates above the olfactory threshold described by Devos et al. (1990). Exceeding this olfactory threshold means that the human nose will detect this odour and may consider this as the first signs of spoilage. Sulphur hydride was produced by all four isolates in a similar concentration (85-91µg/m³) at the end of the storage period (T_9), which is three times higher than the olfactory threshold (25.6 µg/m³). The produced concentrations by each isolate for methyl mercaptan, DMDS, acetone, acetic acid and ammonia is shown in Fig.

5.3. We observed that *Pseudoalteromonas elyakovii* isolate 1G161 produced the highest concentrations of these VOCs and exceeded the olfactory threshold for some of these. *Pseudoalteromonas paragorgicola* (2G68) either did not produce any of the selected VOCs except for sulphur hydride or produced them in extremely low concentrations, which coincides with the species not exceeding 10^8 cfu/g at the end of storage (T_9). It was observed that most of the *Pseudoalteromonas* isolates started producing several compounds after exceeding 10^8 cfu/g (Fig 5.3.). For the other detected VOCs, all produced below the olfactory threshold, isolate 1G161 usually produced the highest concentrations (up to 3 times the concentration of the other *Pseudoalteromonas elakovii* (1G272) and *Pseudoalteromonas nigrifaciens* isolates (1G215)). The maximal concentration of 2-propanol at T_9 was 39µg/m³ for *Pseudoalteromonas elyakovii* (1G161), 24µg/m³ for *Pseudoalteromonas nigrifaciens* (1G215) and 17µg/m³ for *Pseudoalteromonas elakovii* (1G272). For 1,2-butanediol, the maximal concentration was 1804µg/m³ for *Pseudoalteromonas elakovii* (1G161), 3 times higher than the production by *Pseudoalteromonas elakovii* (1G272) (656µg/m³) and *Pseudoalteromonas nigrifaciens* (1G215) (643µg/m³). For butanone, isolate 1G161 (*Pseudoalteromonas elakovii*) produced a maximal concentration of 326µg/m³ at T_9, twice as high as the other 2 isolates (179µg/m³ (*Pseudoalteromonas elakovii* 1G272) and 150µg/m³ (*Pseudoalteromonas nigrifaciens* 1G215)). In contrast to the above mentioned VOCs, *Pseudoalteromonas elyakovii* isolate 1G272 produced the highest concentration of 2-pentanone (168 µg/m³) and ethyl acetate (46 µg/m³), while *Pseudoalteromonas elyakovii* isolate 1G161 and *Pseudoalteromonas nigrifaciens* isolate 1G215 produced a slightly lower concentration of 113 µg/m³ and 89 µg/m³ for 2-pentanone, respectively, and 32 µg/m³ and 24 µg/m³ for ethyl acetate, respectively.

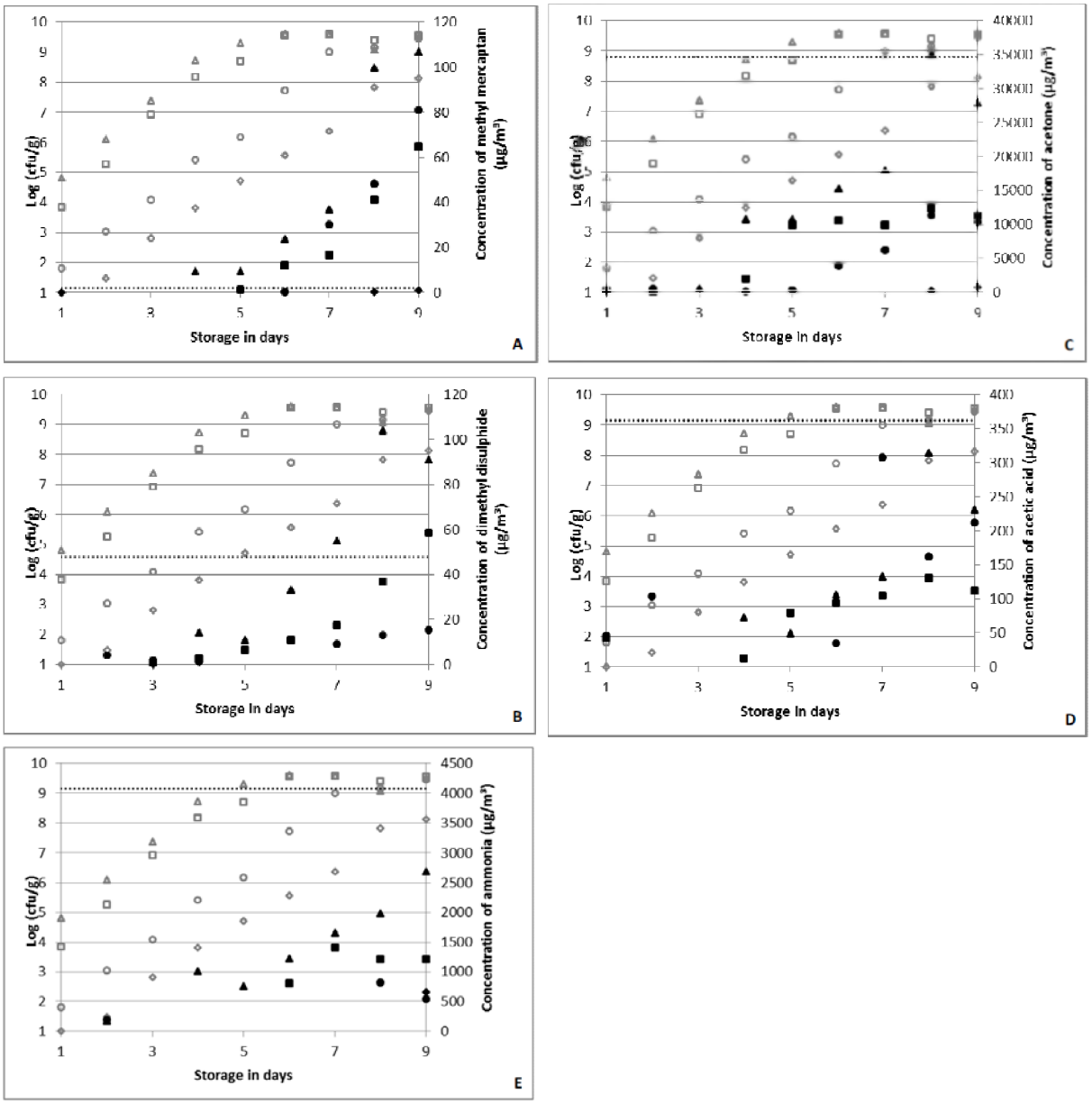

Fig. 5.3. Concentrations (in µg/m³) of (A) methyl mercaptan, (B) dimethyl disulphide, (C) acetone, (D) acetic acid and (E) ammonia produced by *Pseudoalteromonas elyakovii* (1G161 ▲), *Pseudoalteromonas elyakovii* (1G272 ●), *Pseudoalteromonas nigrifaciens* (1G215 ■) and *Pseudoalteromonas paragorgicola* (2G68 ◆) inoculated on sterile brown shrimp are given by filled marks. Each point is the mean of two measurements. This concentration is compared to the days of storage at 4°C in air (X-axis) and the growth of the bacterial isolates in $\log_{10}$ cfu/g (primary Y-axis): The unfilled marks show the bacterial counts: *Pseudoalteromonas elyakovii* (1G161 △), *Pseudoalteromonas elyakovii* (1G272 ○), *Pseudoalteromonas nigrifaciens* (1G215 □) and *Pseudoalteromonas paragorgicola* (2G68 ◇). The olfactory thresholds as described by Devos et al., 1990 are indicated by a dotted line.

IV. Discussion

The objective of this study was to investigate the spoilage potential of the dominant microbiota of brown shrimp, namely species from the genera *Psychrobacter* and *Pseudoalteromonas*. In chapter 4, all species had been isolated from brown shrimp under several different storage conditions. These species were all present during storage on ice, but were particularly numerous at the end of storage, when a TVC of more than 10^8 cfu/g was reached and the shrimp were considered microbiologically spoiled. However, since not all micro-organisms present on fish contribute to spoilage (Dalgaard 1995; Gram and Dalgaard 2002), the spoilage potential of the isolates was determined based on their sensory profile of volatile organic compounds and their biochemical potential based on API ZYM analysis.

Psychrobacter species have been considered to be moderate spoilers, as some produce only weak off-flavours or slightly fishy, musty off-odours (Mejlholm et al. 2005; Prapaiwong et al. 2009; Rodriguez-Calleja et al. 2005). These species lack the important food spoilage attributes such as proteolysis and production of sulphides (Gennari et al. 1992). It was described that they are able to form acids from carbohydrates and show lipase and lecithinase activity but that they do not produce TMA (Garcia-Lopez and Maradona 2000). This study confirms that *Psychrobacter cibarius* and *Psychrobacter maritimus* do not produce significant amounts of VOCs on brown shrimp during storage of 9 days at 4°C, not even when bacterial counts exceeded 10^8 cfu/g. However, two isolates (*Psychrobacter cibarius* 1G232 and *Psychrobacter maritimus* 1G200) did show slight production of TMA during storage at 4°C (Fig 4.2). In comparison to known TMA producers such as *Photobacterium phosphoreum* and *Shewanella putrefaciens*, the produced TMA concentration was considered low. Nevertheless, this slight TMA production by *Psychrobacter* may however explain the slightly fishy off-odour of spoiling seafood described previously (Mejlholm et al. 2005; Prapaiwong et al. 2009). Based on this profile, we may conclude that the isolates of *Psychrobacter* studied here do not significantly contribute to sensorial spoilage and are therefore not SSOs of brown shrimp, but are only weak spoilers as already described in literature (Mejlholm et al. 2005; Prapaiwong et al. 2009; Rodriguez-Calleja et al. 2005). Due to their high abundance during spoilage/shelf life (see chapter 4) however, we cannot conclude that their low importance in spoilage is caused by an inability to compete with common spoilage microorganisms as formulated by Rodriguez-Calleja et al. (2005). Additionally, most of the *Psychrobacter* isolates in this study were able to break down short to medium chain (C4-C8) lipids. This capacity, together with their ability to hydrolyse amino acids (leucine arylamidase) may contribute to spoilage, however, further study is necessary. Lipolytic capacity is a general

characteristic of all species of the genus *Psychrobacter* (Gennari et al. 1992; Yumoto et al. 2003).

In contrast to *Psychrobacter*, the *Pseudoalteromonas* species showed a high spoilage potential. In particular, *Pseudoalteromonas elyakovii* and *Pseudoalteromonas nigrifaciens* appeared to produce high amounts of several VOCs, which may result in sensory rejection of the product. *Pseudoalteromonas paragorgicola* appeared to have a weaker spoilage potential compared to the other species studied. The microorganism did not exceed 10^9 cfu/g, as the other isolates did, after 9 days of storage, which could explain the lower VOC production, since microorganisms start producing VOCs in higher amounts above 10^9 cfu/g (Gram et al. 2002). The two isolates of *Pseudoalteromonas elyakovii* included in this study differed in their VOC production. The isolate 1G161 seemed to produce both more VOCs and higher concentrations of VOCs than isolate 1G272. This strain effect has been described for other microorganisms by Stohr et al. (2001) and Jaffrès et al. (2011).

The production of VOCs by the microorganisms was observed when the total viable count exceeded 10^7 to 10^8 cfu/g, which corresponds to the end of the exponential phase and the beginning of the stationary phase of the growth curves (Fig 5.3.). Gram and Dalgaard (2002) described this as a typical behaviour for SSOs. Our study shows that some of the inoculated *Pseudoalteromonas* species produce especially large amounts of sulphides and acetone. Both volatile compounds are involved in the spoilage process of seafood with sulphides usually involved in the first manifestation of spoilage. It has been described that during aerobic storage, large amounts of ammonia are also formed and that the concentration of acetone, methyl ethyl ketones, dimethyl sulphide and dimethyl disulphide increases continuously (Nychas et al. 2007). Many of these compounds were also formed by most of the *Pseudoalteromonas* isolates studied. In addition to producing several VOCs, all *Pseudoalteromonas* isolates in this study had alkaline phosphatase activity and were also able to break down low to medium chain lipids and hydrolyse proteins and amino acids (i.e., trypsin, α-chymotrypsin and leucine arylamidase). Therefore, not only *Pseudoalteromonas citrea* (Iijima et al. 2009), but also other species within the genus, such as *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas paragorgicola*, have proteolytic potential.

In this study only pure strains were used to observe the spoilage potential of the strains; however, under some conditions, the interaction between spoilage bacteria may influence their growth and metabolism. This interactive behaviour is likely to be important in any food containing various bacterial species during storage (Gram et al. 2002). In brown shrimp, the

microbiota during spoilage is nearly exclusively dominated by *Psychrobacter* and *Pseudoalteromonas* species. Interaction between the species of both genera (e.g. metabiosis) might elevate the spoilage activity of the *Psychrobacter* strains, since *Pseudoalteromonas* has a larger biochemical potential and may create extra nutrients for the growth and metabolic activities of the other microorganisms as observed for other microorganisms by Joffraud et al. (2001).

It also needs to be mentioned that PCR-DGGE analyses between plate swabs and direct DNA extractions showed differences in PCR-DGGE profiles. This may indicate that the overall presence of *Psychrobacter* and *Pseudoalteromonas* species, might be overestimated. This also means that several other microorganisms might contribute to the spoilage of the shrimp. In future research, the VOC profiles of co-inoculated samples as well as a natural contaminated sample should be included to compare with the profiles from the pure strains in this study. However, it has to be considered that autoclaving the shrimp matrix may have an influence on the sensory profile, and may therefore differ from a sensory profile obtained from natural shrimp samples.

In conclusion, this study has contributed to the knowledge concerning the spoilage potential of *Psychrobacter* and *Pseudoalteromonas* isolates/species inoculated as pure cultures on sterile shrimp. The sensory profile results showed that the *Psychrobacter* isolates, identified as three *Psychrobacter cibarius* strains and one *Psychrobacter maritimus* strain, apparently do not contribute to the sensory spoilage of brown shrimp. However, this does not implicate that the *Psychrobacter* spp. do not have any spoilage potential. The off-odours produced during storage of brown shrimp without preservatives appeared to be produced by the isolates identified as *Pseudoalteromonas elyakovii* and *Pseudoalteromonas nigrifaciens*. These isolates produce several volatile compounds (sulphides, acetone, etc.) associated with spoilage.

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PART III

RAY (*RAJA SP.*): THE DOMINANT MICROBIOTA AND THEIR SPOILAGE POTENTIAL

Preamble

Ray belongs to the elasmobranch fish species and is therefore known for the large amount of urea in their cartigilous bones. After catch, this urea is converted to ammonia. Whether this conversion is caused by autolytic or microbial activity is not yet complete certain, however, it is assumed that urease positive bacteria are the most possible cause for the strong ammonia smell shortly after catch. This off-odour makes ray a very perishable seafood product. To date, not much is internationally published about the microbiota during storage/spoilage of elasmobranch fish. In chapter 6 the dominant microbiota of ray during ice storage under various conditions is identified via 16S rRNA, gyr*B and* rpo*B gene sequence analysis. Subsequently, the spoilage potential of several isolates is determined and quantified via mass spectrometry analyses.*

CHAPTER 6

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

Redrafted from:

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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).

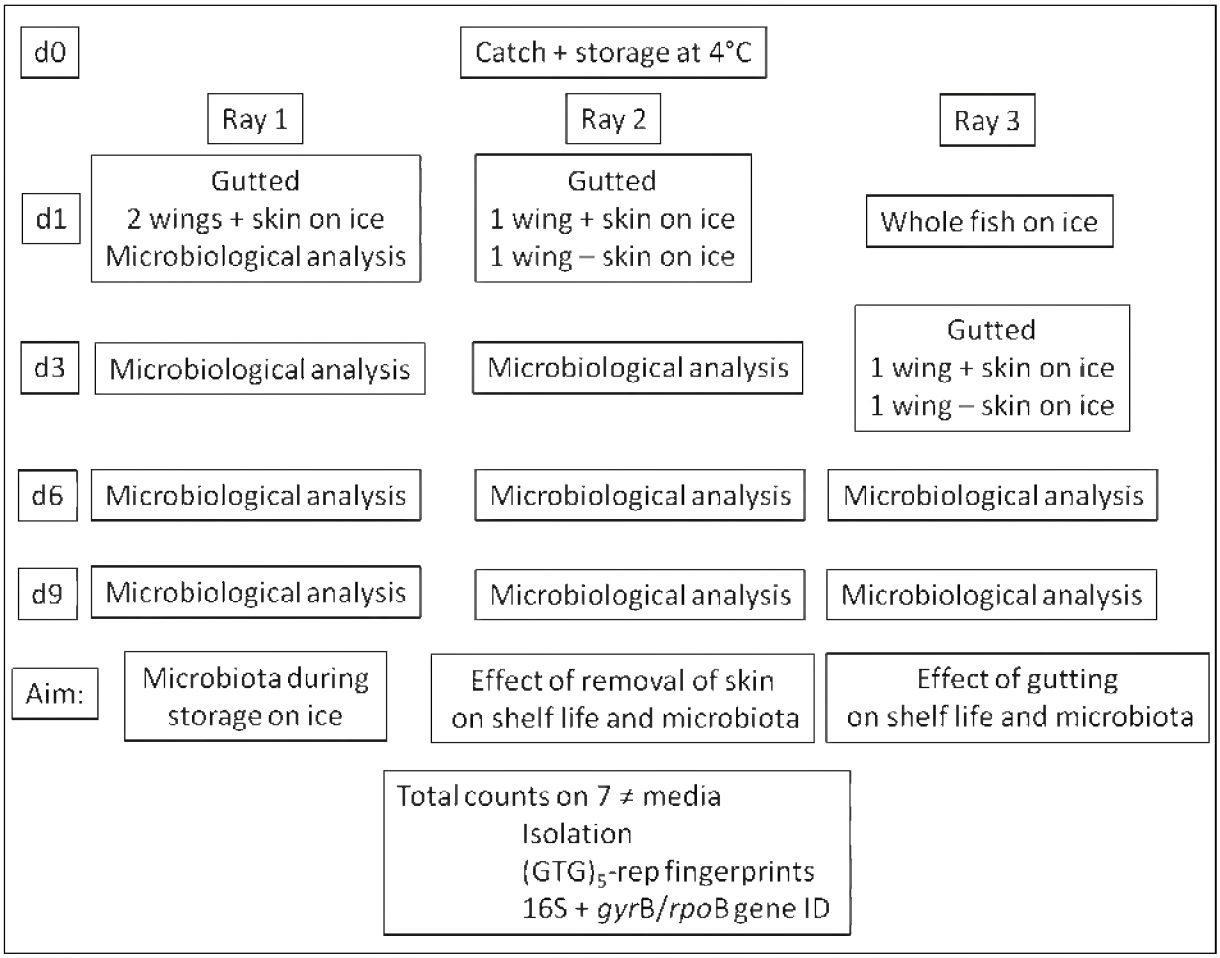

Fig 6.1. Scheme of the three experimental set ups with indication of the time intervals. T_0 = 1 day after catch = arrival at the laboratory, T_1 = after 3 days of aerobic ice storage, T_2 = after 6 days of aerobic ice storage, T_3 = after 9 days of aerobic ice storage.

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.

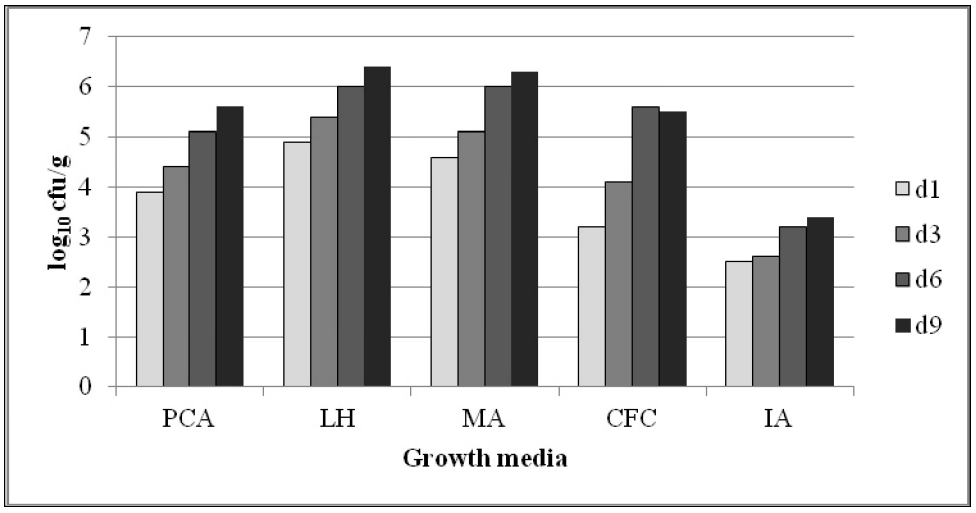

Fig 6.2. Total counts (log_{10} cfu/g) of gutted ray with skin during storage on ice on plate count agar (PCA), Long and Hammer medium (LH), marine agar (MA), *Pseudomonas* cetrimide fucidine cephaloridine (CFC) and Lyngby iron agar (IA). d1= arrival at the laboratory, 1 day after catch and storage on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

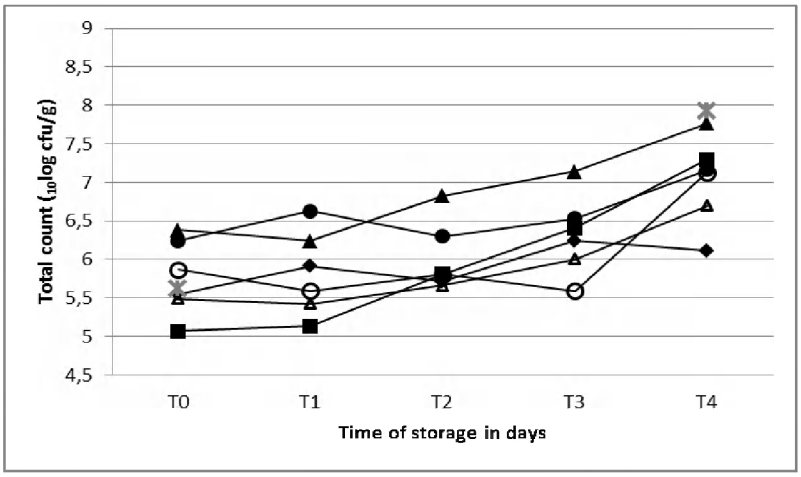

Fig 6.3. Growth of the bacterial inoculated isolates on chilled ray during storage at 4°C in air. The bacterial counts are given in $\log_{10}$ cfu/g. ◆= *Flavobacterium tegetincola* (isolate 3.92), ■= *Shewanella frigidimarina* (isolate 2.175), △= *Psychrobacter cryohalolentis* (isolate 2.256), ▲= *Psychrobacter 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	$C_3H_6^+$

CHAPTER 7

GENERAL CONCLUSIONS, RECOMMENDATIONS AND PERSPECTIVES

Chapter 7. General conclusions, recommendations and perspectives

The fast deterioration of seafood is an important economic loss for the fisheries sector. Since seafood spoilage is mainly caused by microbiological growth and microbiological activity, the study of the microbiota on seafood and the identification of specific spoilage organisms (SSOs) can be considered as an important topic. More knowledge may finally result in an ameliorated quality and shelf life extension of seafood.

To study the microbiological quality of different seafood, a profound fundamental study to detect, identify and characterise the possible spoilage microbiota is necessary. Methodologies were evaluated and optimised and subsequently used to identify the dominant microbiota of brown shrimp (*Crangon crangon*) and ray (*Raja* sp.), and to characterize them to their spoilage potential. In this study both seafood products were not treated with preservatives and air stored.

Spoilage, microbial quality and shelf life of brown shrimp and ray

Today, many researchers are convinced that the use of SSOs or metabolic spoilage associations (MSAs) should be a better approach for seafood quality analysis and shelf life prediction than total viable count (TVC) analysis. SSOs are those microorganisms that grow at certain storage conditions. They grow faster than other microorganisms and produce the metabolites responsible for off-odours and off-flavours resulting in sensory rejection by consumers. Numbers of SSOs and concentrations of their produced metabolites are generally accepted as objective quality indices for shelf life determination (Dalgaard 2000; Erkan and Ozden 2008; Olafsdottir et al. 1997). However, as SSOs are matrix-specific and condition-specific, population shifts will occur depending on the storage conditions. Therefore, thorough research on specific seafood and specific storage conditions is necessary. For several fish species, depending on the conditions of storage, microorganisms such as *Photobacterium*, *Shewanella*, *Pseudomonas* and *Brochothrix* are listed as the SSOs of seafood. However, the possible SSOs on brown shrimp and ray are different from those listed above. Their specific intrinsic (e.g. their large non protein nitrogen (NPN) fraction, high ureum content, etc.) or extrinsic characteristics (traditional processing on board the vessel, possible use of preservatives, etc.) may contribute to this difference in dominant microbiota during storage. This study indicates that for both fishery products the microbiota during aerobic storage on ice predominantly consists of *Psychrobacter* and *Pseudoalteromonas* species. Although on ray, the microbiota during ice storage is co-dominated by *Pseudomonas* species.

Pseudoalteromonas has to date not been mentioned in literature concerning food spoilage. The genus *Pseudoalteromonas* consists of 38 species and two subspecies of which *Pseudoalteromonas haloplanktis* is the type species of the genus. The genus consists of merely marine, strictly aerobic, psychrotolerant, straight or curved rods described between 1995 and 2011. The bacterial strains belonging to the genus, however, have several properties including the production of antifouling agents, the identification as fish and invertebrate pathogens, denitrifying properties, peptide dissolving properties, etc. (Euzeby 1997).

Previously, *Pseudoalteromonas* species belonged to the genus *Alteromonas* until phylogenetic research based on the 16S rRNA gene has shown that the species genetically belonged to a new genus *Pseudoalteromonas* (Gauthier et al. 1995). The genus *Pseudoalteromonas* is closely related to the genus *Shewanella* (Fig. 7.1), which contains species known to be SSOs of seafood. This close relationship might be the cause that the genus *Pseudoalteromonas* was never mentioned before as an SSO in literature. Identification based on phenotypic tests (e.g. API tests), as often performed in food spoilage literature, might incorrectly identify the newly found SSO as belonging to the genus *Shewanella*. In this study, species of the genus *Pseudoalteromonas* were found to actively contribute to spoilage of brown shrimp without preservatives under different storage conditions. This was mainly seen by the production of a large number of volatile organic compounds (VOCs) (sulphides, ammonia etc.) associated to spoilage. The studied isolates also have a large enzymatic capacity. These enzymatic activities might also contribute to spoilage, which is a possible issue for further research.

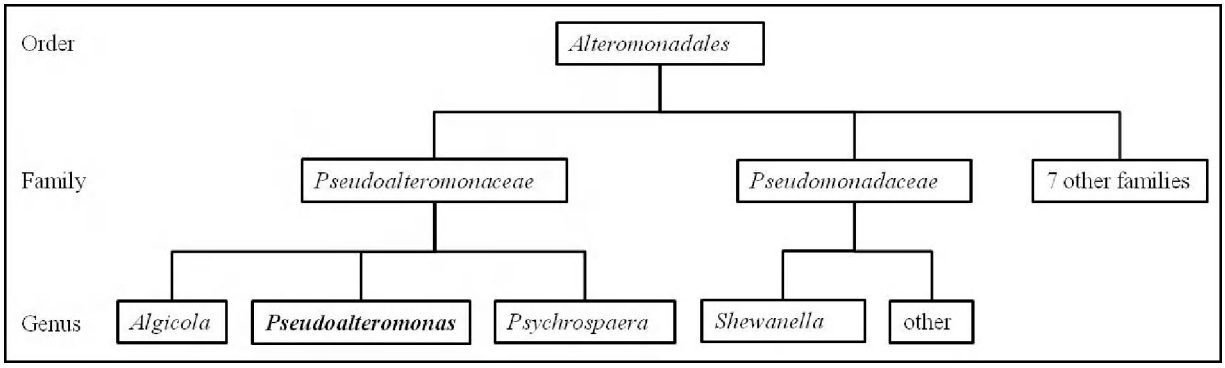

Fig. 7.1. Systematics of the genus *Pseudoalteromonas* in comparison with its' close related genus *Shewanella* (after (Euzeby 1997) - List of Prokaryotic names with Standing in Nomenclature. http://www.bacterio.net).

The colony morphology of all species/isolates of *Pseudoalteromonas* found in this thesis consisted of very slimy big brownish colonies on Long and Hammer medium (LH), transparent or white on marine agar and white-yellowish/white-light orange on *Pseudomonas*

CFC medium. This specific characteristic on LH could be an easy and quick method to recognise the number of *Pseudoalteromonas* spp. on seafood.

The second important genus, *Psychrobacter* counts 33 species and was first described in 1986 by Juni and Heym and was named after "rod that grows at low temperature". The type species is *Psychrobacter immobilis*, a microorganism which has been isolated in large numbers from proteinaceous foods such as fish, processed meat and poultry (Bjorkevoll et al. 2003; Euzeby 1997; Gennari et al. 1992; Juni and Heym 1986). The genus consists merely of aerobic, psychotropic, halotolerant coccobacilli. However, some strains are able to grow under anaerobic conditions when a suitable electron acceptor is present. The taxonomic status of the members of the *Moraxellaceae* family have been constantly under review over the past decades. Phylogenetic research demonstrated their relatedness to *Moraxella* and *Acinetobacter* (Fig. 7.2), both associated with seafood microbiota and spoilage (Gennari et al. 1989). Within the entire family of *Moraxellaceae*, *Moraxella* is the closest related to *Psychrobacter*. However, *Psychrobacter* spp. are considered to have a low spoilage potential because they lack important food spoilage biochemical attributes (Gennari et al. 1992). This study indicated that the spoilage potential of these microorganisms can be matrix- and possibly also strain-dependent. On brown shrimp, *Psychrobacter cibarius* and *Psychrobacter maritimus* did not produce any VOCs. On ray, however, *Psychrobacter cibarius* was able to produce a certain amount of carbonyl sulphides, aldehydes, and ketones.

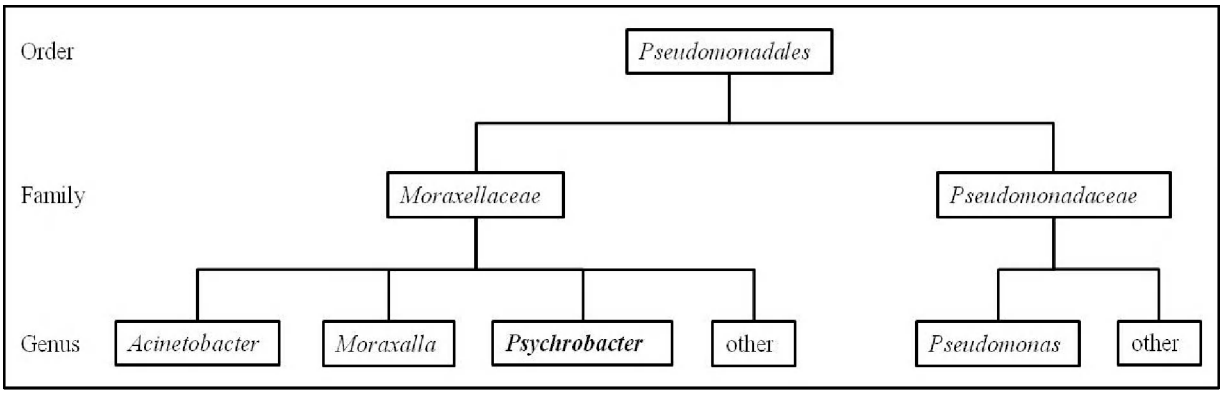

Fig. 7.2. Systematics of the genus *Psychrobacter* in comparison with its' close related genera *Acinetobacter*, *Moraxella* and *Pseudomonas* (after Euzeby 1997) - List of Prokaryotic names with Standing in Nomenclature. http://www.bacterio.net).

Biochemical tests on isolates from ray (Chapter 6) also showed that both *Psychrobacter cibarius* and *Psychrobacter cryohalolentis* are able to degrade urea to ammonia. Members of the genus *Psychrobacter* are considered to grow well on PCA (Blackburn 2006). However, in

this study, some species/isolates were unable to grow on PCA but only on salt containing media such as LH or MA, whereas for other species/isolates the opposite was noticed. The colony morphology of all species within the genus in this thesis grown on LH, MA or PCA was always convex, (transparently) white and relatively small. This colony morphology is however not discriminating enough to identify these microorganisms on culture media.

Future research and opportunities to increase the shelf life of brown shrimp and ray

In order to turn research results into recommendations to the sector, fisheries and seafood processing industry or organisations active in quality control, it is necessary to monitor the microbiota on brown shrimp and ray throughout the year and from different fishing grounds more thoroughly. Only then, it will probably be shown that those microorganisms are indeed persistently present on the stated fishery products. The study of their spoilage potential should also be expanded. On one hand the VOC profile of pure strains on a fishery product should be compared to the profile generated from natural contaminated products as already performed for ray. Also potential interactions between pure strains should be verified, in order to quantify the importance of one SSO within a microbiological association. Furthermore the strains' lipolytic, proteolytic, etc. capacities should be studied more in depth to create a more complete view on their spoilage potential.

Opportunities to increase shelf life might be found in several processing steps of brown shrimp and ray:

One of the methods to extend the shelf life is by optimising the initial microbiological quality of the start product. This could for example be realised through the implementation of a durable fishery technique such as the electrical fishing gear instead of the traditional beam trawling. Since during application of this durable technique the fishing equipments floats above the seabed and the shrimp jump into the net due to electrical pulses, the degree of contamination (e.g. by sand and mud) might be smaller. Also the amount of by-catch will normally be lower as they do not react to the pulses, which also might reduce the initial contamination degree. However, future research is necessary to monitor the effect of different fishing techniques on the initial microbiological quality of the caught raw material.

The current knowledge generated on the microbiota during storage and spoilage of brown shrimp and ray, may form the scientific basis for a profound study and optimisation of the different processing steps [e.g. cooking (only for shrimp), and chilling]. Both *Psychrobacter* and *Pseudoalteromonas* species are associated with the intestinal content of seafood

(Fjellheim et al. 2007; Meziti et al. 2010; Oxley et al. 2002). Further research on their heat resistance, the cooking process (time and temperature), and the maximal temperature and duration inside the shrimp is necessary. Certainly post-cooking contamination can occur and since these microorganisms are present in seawater, contamination has to be prohibited or reduced as much as possible to eliminate or minimise these microorganisms on the product.

Especially for ray it was seen that immediate hygienic gutting of ray could decrease the presence of *Pseudoalteromonas* and *Shewanella putrefaciens*. However the skin of the fish and particularly the slime layer is also a source of contamination (due to the presence of nutrients during the fast onset of decay), it should remain present as long as possible during storage since it also acts as an antibacterial (anti-penetration) layer. To avoid cross-contamination, rinsing with hot water prior to skinning, might influence the microbiological condition and thereby also the shelf life.

The microbiota of ray during ice storage was mainly dominated by several *Pseudomonas* species. *Pseudomonas* spp. actively contribute to spoilage due to their capacity for amino acid degradation resulting in the formation of alcohols, amines, ammonia, esters, ketones and sulphur compounds as described in chapter 2 (Table 2.3). Since *Pseudomonas* spp. are mainly the SSOs of seafood stored under aerobic chilled conditions, a specific packaging technique (e.g. MAP) with specific O_2 and CO_2 levels might ameliorate the shelf life of ray, especially since the other dominant microbiota consists of *Psychrobacter* and *Pseudoalteromonas* species, which are nearly all (strict) aerobic microorganisms. However, a change in storage conditions may induce the growth of other microorganisms present such as lactic acid bacteria, which makes further research essential.

Comments and recommendations for routine laboratory control of seafood

In many routine laboratories, the total (psychotropic) count analysis is determined on general culture media, as an indication of the microbiological quality of seafood.

Based on the evaluation of the four most used general media in seafood quality research (Chapter 3), LH medium, as recommended by the Nordic Committee of Food Analysis, could be assigned as the possible best general culture medium for microbiological analysis on seafood. Since this medium is currently not for sale as a pre-composed dehydrated powder mix, and as a consequence labor intensive to prepare, also MA medium can be used with good results. Chapter 3 showed that the TVC on MA and LH was similar, only some microorganisms were not detected on MA or vice versa. Although many halotolerant

microorganisms and/or SSOs, such as *Photobacterium* spp., *Shewanella* spp., *Pseudoalteromonas* spp. were able to grow on LH, this does not mean that LH was able to give a complete overview of the dominant microbiota or SSOs present during storage. However it is quite clear that the microorganisms mentioned above do not grow on PCA, the current recommended general culture medium by the International Organisation of Standardisation.

Next to the culture media, also the use of microbiological techniques such as the use of pour plating or spread plating and specific incubation temperatures may hamper or facilitate the detection of certain microorganisms (Dalgaard et al. 1997; Reynisson et al. 2009). For instance, the most important SSO of fresh fish, *Photobacterium phosphoreum*, is not easily cultivated compared to other bacteria found in seafood, as the species is vulnerable not only to the salt content in the growth medium, but also to temperature fluctuations (Dalgaard et al. 1997; Emborg et al. 2002).

Therefore, when selecting specifically for a specific SSO, the use of a specific growth medium, such as STAA (Streptomycin-Thallous Acetate-Actidione) agar for *Brochothrix* sp., *Pseudomonas* CFC or Iron agar (black colonies) for *Shewanella* sp. and other H_2S-producing bacteria, is appropriate. When there is no doubt that a specific microorganism can be assigned as the SSO of a seafood product, a specific growth media adapted to its growth requirements and specific (e.g. morphological) characteristics can be developed. The specific detection of the SSO, combined with (the creation of) a mathematical model to calculate the growth of this microorganism such as SSSP (Dalgaard et al. 2002), could then predict the quality and remaining shelf life. Or a species or genus specific molecular detection technique as developed for instance for the genus *Pseudomonas* on seafood by Reynisson et al. 2008, based on the rapid quantitative monitoring by real-time PCR of the *car*A gene, could be applied for this purpose. This method would take less than 5 hours between sampling and results and could therefore be a suitable technique for estimating fast the quality of a fishery product. The last method can however not be considered as ready for commercial use as more research is still necessary.

In general, this thesis indicated that the use of general growth media in this field of fish and fish products analysis should be revised in function of the fish and fish product matrix. A separate analysis method for the microbiological quality of seafood instead of a general method for all food and feed as currently used seems to be more appropriate. Further research

is however necessary, including many more fish species and their SSOs in order to obtain the best (the most complete) general medium or more probable a combination of several media which are able to guarantee a more accurate microbiological quality assessment and shelf life prediction.

Comments and recommendations concerning a molecular approach/research.

The last decade, molecular techniques have been introduced in food microbiology in order to study the present microbiota.

PCR-DGGE (community analysis): In chapters 3 and 4, traditional plating techniques were supplemented with a PCR-DGGE which was used to generate a general overview of the dominant microbiota present and to observe microbiological shifts under certain conditions. The 16S V3 region was chosen as target-region since this region is mostly used in food matrices including seafood (Ercolini 2004; Hovda et al. 2007a, 2007b) and the large in-house experience.

It was seen that PCR-DGGE analysis of plate swabs and direct DNA extraction from the matrix reproduced totally different profiles. It was shown that the absolute dominance of *Pseudoalteromonas* and *Psychrobacter* on shrimp was not present in the profiles obtained via direct DNA extraction. The selection of certain microorganisms based on either nutrients (by conventional plating techniques) or based on the DNA extraction, primer selection and other artefacts (by PCR-DGGE) could be the reason for these differences. For LH medium it was noticed that especially *Pseudoalteromonas* could grow intensively and could overgrow the other microbiota, which was shown in a pronounced dominance of this genus on PCR-DGGE analysis from LH plate swabs. On the other hand, also the PCR-DGGE analysis of DNA extracted directly from the matrix has limitations; not only non-cultivable but also large fractions of non-viable (dead) microorganisms are detected (Cocolin et al. 2007; Ercolini et al. 2001; Olofsson et al. 2007; Rudi et al. 2004).

Although the PCR-DGGE technique is reliable and mostly reproducible (Muyzer 1999), the community fingerprints do not directly give taxonomic information, which is an important limitation (Giraffa and Neviani 2001). Therefore, a sequence analysis of excised and reamplified DNA fragments is necessary. An identification to species level is practically impossible due to the very small fragments generated through PCR-DGGE analysis. Next to the advantages of using PCR-DGGE for microbiological community studies, the technique also suffers from some potential practical biases [e.g. the DNA extraction method used (de

Lipthay et al. 2004), detection limits (Muyzer et al. 1993), selective amplification by the primers (Ercolini et al. 2003), multiple gene copy numbers (de Araujo and Schneider 2008), etc.] as addressed in the Chapters 3 and 4. Another bias linked to PCR-DGGE is the potential co-migration of bands despite sequence variation, this means that one band in the PCR-DGGE pattern may visualise more than one species (Vallaeys et al. 1997). A way to solve this problem is the application of household genes such as *rpo*B or *gyr*B for PCR-DGGE (Dahllof et al. 2000; Peixoto et al. 2002; Renouf et al. 2006; Tacao et al. 2005). However, trial and error in this thesis has shown that several genera need a different primer which demands for an intensive optimisation. In general, it could be hypothesised that the bacterial variation on seafood will possibly be too large to allow the detection of all microorganisms using only one technique. In order to avoid some limiting factors affecting PCR-DGGE analysis, pyrosequencing might be a solution. Pyrosequencing is based on detecting the activity of DNA polymerase with a chemiluminiscent enzyme. It has often been used to observe changes in microbial communities and the technique can discriminate microbial species, types and strains in contrast to PCR-DGGE.

Molecular analysis methods, except for real time PCR methods, are however also often non-quantitative techniques, whereas plating techniques are able to give an idea of the quantity of certain microorganisms. The problem concerning the detection of dead microbiological cells in all molecular methods can only be solved by using reverse transcriptase PCR techniques, which needs intensive optimisation. The largest disadvantage of any molecular technique for detection is the inability to obtain extra information e.g. the spoilage capacity of the microorganisms, since they are not cultivated/isolated. The optimal technique, conventional or molecular or a combination of both, will therefore depend on the aim of the experiment.

Molecular identification: After detection and cultivation of microorganisms or SSOs of seafood by conventional plating, it can, depending on the purpose of the analysis, be important that they are correctly identified to species level. This is still too often performed by phenotypic testing which lacks sensitivity due to the large diversity and the variety of especially marine microbiota. Biochemical test kits (e.g. API, BIOLOG) are often not adapted for identification of these microorganisms (except for the already known SSOs) and would assign the isolate a wrong identity. Molecular techniques are more appropriate to this matter, but also have their limitations and disadvantages. Since molecular identification techniques based on sequencing are quite expensive and the colony morphology of identical isolates may

vary depending on the selected media and incubation parameters, a de-replication (grouping the identical isolates at a certain taxonomic level) of all isolates may be necessary. In order to de-replicate the large number of isolates, rep-PCR fingerprinting was used in this thesis. Comparing REP, BOX and $(GTG)_5$ primers showed that only $(GTG)_5$ – rep fingerprinting was useful based on the number of bands generated for marine microorganisms in this study. $(GTG)_5$-rep fingerprinting is a powerful technique that allows the analysis of a high amount of isolates from different taxonomic groups without prior genotypic knowledge with a high taxonomic resolution (between species and strain level) (Gevers et al. 2001; Versalovic et al. 1991). This high taxonomic resolution could not be applied to all genera studied in this thesis. It was observed especially for the genus *Psychrobacter* that isolates clustering together with a Pearson's correlation coefficient above 80% were not identified as the same species based on 16S and *gyr*B gene sequencing. This made provisionary identification based on the rep-profiles with type strains not reliable. Therefore several isolates from large clusters were always sequenced in order to avoid incorrect identification.

However, identification based on the 16S rRNA gene is considered to be the golden standard in prokaryotic systematics (Hillis and Dixon 1991; Stackebrandt and Ebers, 2006; Woese 1987). Identification based on the partial or nearly full 16S rRNA gene of especially four genera (*Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* and *Shewanella*) in this thesis resulted in species complexes with a similarity above 99% with several species. As described by Stackebrandt and Ebers (2006) a similarity above 98.7% is needed to be identified as the same species, a similarity which can be correlated to 70% DNA-DNA hybridization. 16S rRNA gene sequencing thereby limited the identification of the above listed genera. Sequence analysis of household genes which have a wider sequence variation and evolve more rapidly, such as *rpo*B or *gyr*B might solve this problem (Giraffa and Neviani 2001; Palys et al. 1997; Yamamoto and Harayama 1995). For the genus *Pseudomonas*, it was chosen to use the *rpo*B gene for identification due to the large in-house experience (De Jonghe et al. 2011; Marchand et al. 2009) and literature search (Tayeb et al. 2005). However, even with *rpo*B sequencing the identification of isolates remained indefinite. In general, it is known that the classification of *Pseudomonas* strains is problematic due to the lack of an accurate taxonomic system (Yamamoto et al. 2000). Methods, such as DNA-rRNA hybridization, DNA-DNA hybridization, direct comparison of rRNA sequences have been applied in order to establish the phylogenetic relationships between new isolates and previously defined taxa (De Vos et

al. 1989; Palleroni et al. 1973; Ursing 1986). However, identification remains often difficult (Tryfinopoulou et al. 2002). This problem is not limited for the genus *Pseudomonas*.

In general, thorough studies for the identification and systematics of marine microorganisms is lacking. Not only are many species associated to seafood spoilage often identified by phenotypical techniques, also a large amount of species are not yet described (novel species) or lack profound identification.

For the genera *Shewanella*, *Psychrobacter*, and *Pseudoalteromonas*, the *gyr*B gene was used for species identification. Discrepancies between 16S and *gyr*B genes were often found and could perhaps be assigned to the presence of novel species. For the genus *Shewanella* several isolates were identified as a *Shewanella frigidimarina – vesiculosa – livingstonensis* species complex based on 16S identification. Phylogenetic tree analysis with *gyr*B showed a higher similarity with *Shewanella frigidimarina*. However, a sequence comparison with *Shewanella arctica* sp. nov. (Kim et al. 2011), a recent novel species within the genus, shows that the isolates, identified as *Shewanella frigidimarina* (*gyr*B) in this thesis, have a 99.9 % similarity with the novel species based on 16S. The discrepancies between the genes can therefore possibly be explained by species closely related to currently known species, but which are in fact novel species. For the genera *Psychrobacter* and *Pseudoalteromonas*, as described in chapter 4, phylogenetic tree analysis showed that for 16S sequences also the species type strains showed a very low genetic distance between them, which could be resolved by using *gyr*B. The use of household genes, in combination with 16S, might therefore possibly become more useful in prokaryotic systematics.

REFERENCES

References

Adams, R., Farber, L. and Lerke, P., 1964 Bacteriology of spoilage of fish muscle. II. Incidence of spoilers during spoilage. Applied Microbiology 12, 277-279.

Adams, M.R. and Nicolaides, L., 1997 Review of the sensitivity of different foodborne pathogens to fermentation. Food control 8, 227-239.

Akankwasa, A. (1998) The effect of delayed icing and gutting on the quality of freshwater arctic charr (*Salvelinus alpinus* L.). pp. 1-17.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller W., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Alvarez, R.J., 1982. Role of *Planococcus citreus* in the spoilage of *Pennaeus* shrimp. Zentralblatt fur bakterologie, parasitenkunde, infektionkrankheiten und hygiene I ,503-512.

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C. and Guyot, J.P., 1999 Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Applied and Environmental Microbiology 65, 5464-5473.

Anonymous, 1986. ICMSF: sampling for microbiological analysis: principles and specific applications. In: Microorganisms in Foods, second ed.. University of Toronto Press, Buffalo, pp. 127-278.

Anonymous, 1995. European parliament and council directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. Official Journal of the European Community No L-61, 18. 3. 1995.

Anonymous, 1996. Council Regulations (EC) No 2406/96 of 26 November 1996 'Laying down common marketing standards for certain fishery products. Official Journal of the European Community No L-334/2.23.12.1996.

Anonymous, 2006. Fishery statistics. In Food and Agriculture Organization of the United Nations yearbook 2004 ed. FAO pp. 139-306. Rome: FAO.

Anonymous, 2009a. De Belgische zeevisserij: aanvoer en besomming 2009. pp. 1-109. Vlaamse Overheid - Landbouw en Visserij Dept.

Anonymous, 2009b. Report of the working group on *Crangon* fisheries and life history. ICES CM 2009/LRC:07. 2009.

Anonymous. 2011. The North Sea brown shrimp fisheries. European Parliament - Directorate-General for Internal Policies – Policy Department Structural and Cohesion policies B - Fisheries. ISBN 978-92-823-3430-0, Brussels,1-106 (http://www.europarl.europa.eu/studies).

Arthur, C.L. and Pawliszyn, J., 1990. Solid phase micro extraction with thermal desorption using fused silica optical fibres. Analytical Chemistry 62, 2145-2148.
Atlas, R.M, 2006. Handbook of microbiological media for the examination of food, second ed. Fl.: CPC Press - Taylor and Francis Group, Boca Raton.

Austin, B., 2006 The bacterial microflora of fish, revised. The scientific world journal 6, 931-945.

Austin, B. and Austin, D.A., 1999. Bacterial fish pathogens: disease of farmed and wild fish. Praxis Publishing, Chichester, UK.

Bagge-Ravn, D., Ng, Y., Hjelm, M., Christiansen, J.N., Johansen, C. and Gram, L., 2003. The microbiological ecology of processing equipment in different fish industries-analysis of the microflora during processing and following cleaning and disinfection. International Journal of Food Microbiology87, 239-250.

Balamatsia, C.C., Patsias, A., Kontominas, M.G. and Savvaidis, I.N., 2007. Possible role of volatile amines as quality-indicating metabolites in modified atmosphere-packaged chicken fillets: Correlation with microbiological and sensory attributes. Food Chemistry 104, 1622-1628.

Baumann, P. R. & Schubert, H. W. (1984). Family II: *Vibrionaceae* . In N. R. Krieg & J. G. Holt (Eds.), Bergey's manual of systematic bacteriology (1th ed., pp. 516-517). Baltimore: Williams and Wilkins.

Bekaert, K., Maryssael, P. and Desmyter, B. 2007. Handleiding voor de beoordeling van de versheid van vis volgens de Kwaliteit Index Methode. pp. 1-43. Oostende, Belgium: V.U. Rederscentrale.

Belchior, S.G.E. and Vacca, G., 2006. Fish protein hydrolysis by a psychotolerant marine bacterium isolated from the gut of hake (*Merluccius hubbsi*). Canadian Journal of Microbiology 52, 1266-1271.

Bernardet, J. F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., and Vandamme, P. 1996. Cutting a gordian knot: Emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom nov (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). International Journal of Systematic Bacteriology, 46, 128-148.

Bilinski, E., Jonas, R.E.E. and Peters, M.D., 1983. Factors controlling the deterioration of the spiny dogfish *Squalus acanthias*, during iced storage. Journal of Food Science 48, 808-812.

Bjorkevoll, I., Olsen, R.L. and Skjerdal, O.T., 2003. Origin and spoilage potential of the microbiota dominating genus *Psychrobacter* in sterile rehydrated salt-cured and dried salt-cured cod (*Gadus morhua*). International Journal of Food Microbiology, 84, 175-187.

Blackburn, C. 2006. Food spoilage microorganisms. Boca Raton, FL: CRC Press.

Bon, J., 1996. Garnalenverwerking onder de loep. De Ware(n)-Chemicus 26, 255-261.

Borch, E., Kant-Muermans, M.L. and Blixt, Y., 1996. Bacterial spoilage of meat and cured meat products. International Journal of Food Microbiology33, 103-120.

Boskou, G. & Debevere, J. 1996. Optimum medium for total viable counts of spoilage of cod fillets packed under modified atmospheres. In (pp. 1593-1603). Brugge: Med. Fac. Landbouw, Univ. Gent, 61/4a.

Boskou, G. and Debevere, J., 1997. Reduction of trimethylamine oxide by *Shewanella* spp. under modified atmospheres in vitro. Food Microbiology 14, 543-553.

Bowman, J.P., 2006. The Genus *Psychrobacter*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E. (Eds.), The Prokaryotes - A handbook on the biology of bacteria. 3th Ed., vol. 6. Springer New York, pp. 920-930.

Bowman, J. P., McCammon, S. A., Nichols, D. S., Skerratt, J. H., Rea, S. M., Nichols, P. D. et al. 1997. *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5 omega 3) and grow anaerobically by dissimilatory Fe(III) reduction. International Journal of Systematic Bacteriology, 47, 1040-1047.

Bozal, N., Montes, M. J., Tudela, E., and Guinea, J. 2003. Characterization of several *Psychrobacter* strains isolated from Antarctic environments and description of *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov. International Journal of Systematic and Evolutionary Microbiology, 53, 1093-1100.

Braun, P. and Sutherland, J.P., 2003. Predictive modelling of growth and enzyme production and activity by a cocktail of *Pseudomonas* spp., *Shewanella putrefaciens* and *Acinetobacter* sp. International Journal of Food Microbiology86, 271-282.

Brosius, J., Palmer, M. L., Kennedy, P. J., & Noller, H. F. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proceedings of the National Academy of Sciences, 75, 4801-4805.

Brown, W. D. 1986. Fish muscle as food. In Muscle as food ed. Bechtel,P.J. pp. 405-451. Orlando, Fla.: Academic Press.

Buchanan, R.L. and Bagi, L.K., 1997. Microbiological competition: Effect of culture conditions on the suppression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*. Journal of Food Protection 60, 254-261.

Castell, C.H. and Mapplebeck, E.G., 1952. The importance of *Flavobacterium* in fish spoilage. Journal of fisheries research board Canada 9, 148-156.

Castell, C.H. and Snow, J.M., 1949. The effect of pH on the enzymatic reduction of trimethylamine oxide. Journal of the Fisheries Research Board of Canada 7, 561-562.

Chai, T., Chen, C., Rosen, A. and Levin, R., 1968. Detection and incidence of specific species of spoilage bacteria on fish - II. Relative incidence of *Pseudomonas putrefaciens* and fluorescent pseudomonads on haddock fillets. Applied Microbiology 16, 1738-1741.

Chinivasagam, H.N., Bremner, H.A., Wood, A.F. and Nottingham, S.M., 1998. Volatile components associated with bacterial spoilage of tropical prawns. International Journal of Food Microbiology 42, 45-55.

Choi, J.H., Im, W.T., Liu, Q.M., Yoo, J.S., Shin, J.H., Rhee, S.K. and Roh, D.H., 2007. *Planococcus donghaensis* sp. nov., a starch-degrading bacterium isolated from the East Sea, South Korea. International Journal of Systematic and Evolutionary Microbiology 57, 2645-2650.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B.K. and Lim, Y.W., 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57, 2259-2261.

Chung, H.Y. and Cadwallader, K.R., 1993. Volatile components in blue crab (*Callinectes sapidus*) meat and processing by-product. Journal of Food Science 58, 1203-1207.

Cobb, B.B. and Mathews, J.J., 1973. Proposed New Test for Aptitude Screening of Air-Traffic Controller Applicants. Aerospace Medicine 44, 184-189.

Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I. and Beimfohr, C., 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. International Journal of Food Microbiology120, 100-109.

Dahllof, I., Baillie, H. and Kjelleberg, S., 2000. *rpo*B-based microbiological community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. Applied and Environmental Microbiology 66, 3376-3380.

Dalgaard, P., 1995a. Modelling of microbiological activity and prediction of shelf life for packed fresh fish. International Journal of Food Microbiology26, 305-317.

Dalgaard, P., 1995b. Qualitative and quantitative characterization of spoilage bacteria from packed fish. International Journal of Food Microbiology26, 319-333.

Dalgaard, P., 2000. FLAIR-FLOW Europe Technical Manual F-FE 380 A - 00.

Dalgaard, P., 2006. Microbiology of marine muscle foods. In Handbook of Food Science, Technology and Engineering ed. Hui,Y.H. pp. 1-20. Boca Raton: CRC Press.

Dalgaard, P., Buch, P. and Silberg, S., 2002. Seafood Spoilage Predictor--development and distribution of a product specific application software. International Journal of Food Microbiology 73, 343-349.

Dalgaard, P., Christiansen, T.J. and Huss, H.H., 1997. Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products. Letters in Applied Microbiology 24, 373-378.

Dalgaard, P., Gram, L. and Huss, H.H., 1993. Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres. International Journal of Food Microbiology19, 283-294.

Dalgaard, P. and Jorgensen, L.V., 2000. Cooked and brined shrimps packed in a modified atmosphere have a shelf-life of > 7 months at 0°C, but spoil in 4-6 days at 25°C. International Journal of Food Science and Technology 35, 431-442.

Dalgaard, P., Vancanneyt, M., Euras, V.N., Swings, J., Fruekilde, P. and Leisner, J.J., 2003. Identification of lactic acid bacteria from spoilage associations of cooked and brined shrimps stored under modified atmosphere between 0 degrees C and 25 degrees C. Journal of Applied Microbiology, 94, 80-89.

Dams, E., Hendriks, L., Vandepeer, Y., Neefs, J.M., Smits, G., Vandenbempt, I. and Dewachter, R., 1988. Compilation of small ribosomal-subunit RNA sequences. Nucleic Acids Research 16, R87-R173.

Dang, T.D.T., Vermeulen, A., Ragaert, P. and Devlieghere, F., 2009. A peculiar stimulatory effect of acetic and lactic acid on growth and fermentative metabolism of *Zygosaccharomyces bailii*. Food Microbiology 26, 320-327.

de Araujo, J.C. and Schneider, R.P., 2008. PCR-DGGE with genomic DNA: Suitable for detection of numerically important organisms but not for identification of the most abundant organisms. Water Research 42, 5002-5010.

De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. and Heyndrickx, M., 2011. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Applied and Environmental Microbiology 77, 460-470.

de Lipthay, J.R., Enzinger, C., Johnsen, K., Aamand, J. and Sorensen, S.J., 2004. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. Soil Biology & Biochemistry 36, 1607-1614.

De Vos, P., Van Landschoot, A., Segers, P., Tytgat, R., Gillis, M., Bauwens, M., Rossau, R., Goor, M., Pot, B., Kersters, K., Lizzaraga, P. and De Ley, J. 1989. Genotyping relatioships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hydridization. International Journal of Systematic Bacteriology 39:35-49.

Devos, M., Patte, F., Rouault, J., Laffort, P. and Van Gemert, L. J. 1990. Standardized human olfactory thresholds. Oxford: IRL Press at Oxford University Press.

Diehl, J.F., 1992. Food irradiation: is it an alternative to chemical preservatives? Food Additives and Contaminants 9, 409-416.

Donovan, C.J., Garduno, R.A., Kalmokoff, M., Ku, J.C., Quilliam, M.A. and Gill, T.A., 2009. *Pseudoalteromonas* bacteria are capable of degrading paralytic shellfish toxins. Applied and Environmental Microbiology 75, 6919-6923.

Drosinos, E.H. and Board, R.G., 1994. Metabolic activities of *Pseudomonads* in batch cultures in extract of minced lamb. Journal of Applied Bacteriology, 77, 613-620.

Duflos, G., Coin, V.M., Cornu, M., Antinelli, J.F. and Mallet, P., 2006. Determination of volatile compounds to characterize fish spoilage using headspace/mass spectrometry and solid-phase microextraction/gas chromatography/mass spectrometry. Journal of the Science of Food and Agriculture 86, 600-611.

Duun, A.S. and Rustad, T., 2008. Quality of superchilled vacuum packed Atlantic salmon (*Salmo salar*) fillets stored at -1.4 and -3.6 degrees C. Food Chemistry 106, 122-131.

Dykes, G.A., Vegar, M. and. Vanderlinde, P.B., 2003. Quantification of *Listeria* spp. contamination on shell and flesh of cooked black tiger prawns (*Penaeus monodon*). Letters in Applied Microbiology 37, 309-313.

Edwards, R.A., Dainty, R.H. and Hibbard, C.M., 1987. Volatile compounds produced by meat pseudomonads and related reference strains during growth on beef stored in air at chill temperatures. Journal of Applied Bacteriology 62, 403-412.

Einarsson, H. and Lauzon, H.L., 1995. Biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 61, 669-676.

Elliot, R.P., 1952. Reduction of trimethylamine oxide in dogfish flesh. Food Research 17, 225-233.

Elotmani, F., Assobhei, O., Revol-Junelles, A.M. and Milliere, J.B., 2004. Microflora of fresh and ice-stored sardines (*Sardina pilchardus*) from the Moroccan Atlantic coast. Ciencias Marinas 30, 627-635.

Emborg, J., Laursen, B.G., Rathjen, T. and Dalgaard, P., 2002. Microbiological spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmo salar*) at 2 degrees C. Journal of Applied Microbiology 92, 790-799.

Engelbrecht, K., Jooste, P.J. and Prior, B.A., 1996. Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. The South African Journal of Food Science and Nutrition 8, 66-71.

Ercolini, D., 2004 PCR-PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297-314.

Ercolini, D., Hill, P.J. and Dodd, C.E., 2003. Bacterial community structure and location in Stilton cheese. Applied and Environmental Microbiology 69, 3540-3548.

Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S., 2001. The potential of a polyphasic PCR-PCR-DGGE approach in evaluating microbiological diversity of natural whey cultures for water-buffalo Mozzarella cheese production: bias of culture-dependent and culture-independent analyses. Systematics in Applied Microbiology 24, 610-617.

Erkan, N., 2007. Sensory, chemical, and microbiological attributes of sea bream (*Sparus aurata*): Effect of washing and ice storage. International Journal of Food Properties 10, 421-434.

Erkan, N. and Ozden, O., 2008. Quality assessment of whole and gutted sardines (*Sardina pilchardus*) stored in ice. International Journal of Food Science and Technology 43, 1549-1559.

Euzeby, J.P., 1997. List of bacterial names with standing in nomenclature: A folder available on the Internet. International Journal of Systematic Bacteriology 47, 590-592.

Fagan, J.D., Gormley, T.R. and Ui Mhuircheartaigh, M.M., 2004. Effect of modified atmosphere packaging with freeze-chilling on some quality parameters of raw whiting, mackerel and salmon portions. Innovative Food Science and Emerging Technologies 5, 205-214.

Farmer, J. J. & Hickman-Brenner, F. W. 2006. The Genera *Vibrio* and *Photobacterium*. In M.Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), The Prokaryotes - A Handbook on the Biology of Bacteria (3 ed., pp. 508-563). New York: Springer.

Feldhusen, F., 2000. The role of seafood in bacterial foodborne diseases. Microbes and Infection 2, 1651-1660.

Fennema, O. R., Powrie, W. D. and Marth, E. H. 1973. Low-temperature preservation of foods and living matter. New York: Dekker.

Finne, G. 1982. Enzymatic ammonia production in shrimp held on ice. In Chemistry and biochemistry of marine food products ed. Martin,R.E., Hebard,C.E. and Ward,D.R. pp. 323-331. Westport, Conn.: AVI Publishing Co. Inc.

Finne, G. 1992. Non-protein nitrogen compounds in fish and shellfish. In Advances in seafood biochemistry ed. Flick,G. and Martin,R. pp. 393-401. Lancaster, PA: Technomic Publishing Compagy Inc.

Fjellheim, A.J., Playfoot, K.J., Skjermo, J. and Vadstein, O., 2007. *Vibrionaceae* dominates the microflora antagonistic towards *Listonella anguillarum* in the intestine of cultured Atlantic cod (*Gadus morhua* L.) larvae. Aquaculture 269, 98-106.

Flamm, R. K., Hinrichs, D. J., & Thomashow, M. F. 1984. Introduction of pAMβ1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. Infection and immunity, 44, 157-161.

Floros, J. D. and Matsos, K. I. 2005. Introduction to modified atmosphere packaging. In Innovations in Food Packaging pp. 159-172. Elsevier Ltd.

Fox, G.E., Wisotzkey, J.D. and Jurtshuk, P., 1992. How close is close - 16S ribosomal RNA sequence identity may not be sufficient to guarantee species identity. International Journal of Systematic Bacteriology 42, 166-170.

Freeman, L.R., Silverman, G.J., Angelini, P., Merritt, C. and Esselen, W.B., 1976. Volatiles produced by microorganisms isolated from refrigerated chicken at spoilage. Applied and Environmental Microbiology 32, 222-231.

Gauthier, G., Gauthier, M. and Christen, R., 1995. Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. International Journal of Systematic Bacteriology 45, 755-761.

Garcia-Lopez, M.-L. and Maradona, M. P. 2000. *Psychrobacter*. In Encyclopaedia of Food Microbiology ed. Robinson,R.K., Batt,C.A. and Patel,P.D. pp. 1875-1882. London: Acedemic Press.

Gennari, M., Alacquac, G., Ferri, F. and Serio, M., 1989. Characterization by conventional methods and genetic transformation of *Neisseriaceae* (genera *Psychrobacter* and *Acinetobacter*) isolated from fresh and spoiled sardines. Food Microbiology 6, 199-210.

Gennari, M. and Dragotto, F., 1992. A study of the incidence of different fluorescent *Pseudomonas* species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil and water. Journal of Applied Bacteriology 72, 281-288.

Gennari, M., Parini, M., Volpon, D. and Serio, M., 1992. Isolation and characterization by conventional methods and genetic-transformation of *Psychrobacter* and *Acinetobacter* from fresh and spoiled meat, milk and cheese. International Journal of Food Microbiology 15, 61-75.

Gennari, M., Tomaselli, S. and Cotrona, V., 1999. The microflora of fresh and spoiled sardines (*Sardina pilchardus*) caught in Adriatic (Mediterranean) Sea and stored in ice. Food Microbiology 16, 15-28.

Gevers, D., Huys, G. and Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. Fems Microbiology Letters 205, 31-36.

Gill, C.O., 1976. Substrate limitation of bacterial growth at meat surfaces. Journal of Applied Bacteriology 41, 401-410.

Gill, C. O. 1982. Microbiological interaction with meats. In Meat microbiology ed. Brown,M.H. pp. 225-264. New York: Applied Science Publishers.

Gillespie, N.C. and Macrae, I.C., 1975. The bacterial flora of some Queensland fish and its ability to cause spoilage. Journal of Applied Bacteriology 39, 91-100.

Giraffa, G. and Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbiological communities in food-associated ecosystems. International Journal of Food Microbiology67, 19-34.

Goncalves, A.C., Lopez-Caballero, M.E. and Nunes, M.L., 2003. Quality changes of deepwater pink shrimp (*Parapenaeus longirostris*) packed in modified atmosphere. Journal of Food Science 68, 2586-2590.

Gram, L., 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. Applied and Environmental Microbiology 59, 2197-2203.

Gram, L., 1994. Siderophore-mediated iron sequestering by *Shewanella putrefaciens*. Applied and Environmental Microbiology 60, 2132-2136.

Gram, L. and Dalgaard, P., 2002. Fish spoilage bacteria--problems and solutions. Current Opinion in Biotechnology, 13, 262-266.

Gram, L. and Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology33, 121-137.

Gram, L. and Huss, H. H. 2000. Fresh and processed fish and shellfish. In The microbiological safety and quality of foods ed. Lund,B.M., Baird-Parker,A.C. and Gould,G.W. pp. 472-506. Gaithersburg, Md.: Aspen Publishers, Inc.

Gram, L. and Melchiorsen, J., 1996. Interaction between fish spoilage bacteria *Pseudomonas* sp. and *Shewanella putrefaciens* in fish extracts and on fish tissue. Journal of Applied Bacteriology 80, 589-595.

Gram, L., Trolle, G. and Huss, H.H., 1987. Detection of Specific Spoilage Bacteria from Fish Stored at Low (0-Degrees-C) and High (20-Degrees-C) Temperatures. International Journal of Food Microbiology 4, 65-72.

Gram, L., Wedellneergaard, C. and Huss, H.H., 1990. The bacteriology of fresh and spoiling Lake Victorian Nile perch (*Lates niloticus*). International Journal of Food Microbiology 10, 303-316.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B. and Givskov, M., 2002. Food spoilage-interactions between food spoilage bacteria. International Journal of Food Microbiology 78, 79-97.

Grigorakis, K., Taylor, K.D.A. and Alexis, M.N., 2003. Seasonal patterns of spoilage of ice-stored cultured gilthead sea bream (*Sparus aurata*). Food Chemistry 81, 263-268.

Grützmacher, H. F. 1999. Fragmentation in mass spectrometry. In Encyclopedia of spectroscopy and spectrometry ed. Lindon,J.C., Tranter,G.E. and Holmes,J.L. London: Academic Press.

Hansen, A.A., Morkore, T., Rudi, K., Olsen, E. and Eie, T., 2007. Quality changes during refrigerated storage of MA-packaged pre-rigor fillets of farmed Atlantic cod (*Gadus morhua* L.) using traditional MAP, CO_2 emitter, and vacuum. Journal of Food Science 72, M423-M430.

Hao, M.V. and Komagata, K., 1985. A new species of *Planococcus*, *P. kocurii* isolated from fish, frozen foods and fish curing brine. The Journal of General and Applied Microbiology 31, 441-455.

Haugen, J.E., Chanie, E., Westad, F., Jonsdottir, R., Bazzo, S., Labreche, S., Marcq, P., Lundby, F. and Olafsdottir, G., 2006. Rapid control of smoked Atlantic salmon (*Salmo salar*) quality by electronic nose: Correlation with classical evaluation methods. Sensors and Actuators B-Chemical 116, 72-77.

Haugen, J.E. and Kvaal, K., 1998. Electronic nose and artificial neural network. Meat Science 49, S273-S286.

Haugen, J.E. and Undeland, I., 2003. Lipid oxidation in herring fillets (*Clupea harengus*) during ice storage measured by a commercial hybrid gas-sensor array system. Journal of Agricultural and Food Chemistry 51, 752-759.

Herbert, R.A., Hendriff, M.S., Gibbson, D.M. and Shewan, J.M., 1971. Bacteria active in the spoilage of certain seafoods. Journal of Applied Bacteriology 34, 41-50.

Herbert, R.A. and Shewan, J.M., 1976. Roles played by bacterial and autolytic enzymes in the production of volatile sulphides in spoiling North Sea cod (*Gadus morhua*). Journal of the Science of Food and Agriculture 27, 89-94.

Hillis, D.M. and Dixon, M.T., 1991. Ribosomal DNA - molecular evolution and phylogenetic inference. Quarterly Review of Biology 66, 411-453.

Hovda, M.B., Lunestad, B.T., Sivertsvik, M. and Rosnes, J.T., 2007a. Characterisation of the bacterial flora of modified atmosphere packaged farmed Atlantic cod (*Gadus morhua*) by PCR-PCR-DGGE of conserved 16S rRNA gene regions. International Journal of Food Microbiology117, 68-75.

Hovda, M.B., Sivertsvik, M., Lunestad, B.T., Lorentzen, G. and Rosnes, J.T., 2007b. Characterisation of the dominant bacterial population in modified atmosphere packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-PCR-DGGE. Food Microbiology, 24, 362-371.

Hugenholtz, P., Goebel, B.M. and Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology 180, 4765-4774.

Huidobro, A., Lopez-Caballero, M.E. and Mendes, R., 2002. Onboard processing of deepwater pink shrimp (*Parapenaeus longirostris*) with liquid ice: Effect on quality. European Food Research and Technology 214, 469-475.

Huss, H. H. 1995. Assurance of seafood quality. Rome, Italy: United Nations Food and Agriculture Organization.

Huss, H. H. 1995. Quality and changes in fresh fish. Food and agriculture organization of the United Nations, FAO fisheries technical paper - 348, 1-195.

Huss, H.H., Dalgaard, P. and Gram, L., 1997. Microbiology of fish and fish products. Development in food science 38, 413-430.

Iijima, S., Washio, K., Okahara, R. and Morikawa, M., 2009. Biofilm formation and proteolytic activities of *Pseudoaltermonas* bacteria that were isolated from fish farm sediments. Microbiological Biotechnology 2, 361-369.

International Organization for Standardization. ISO 17410. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of psychrotropic microorganisms. 17410:2001

Ivanova, E.P., Kiprianova, E.A., Mikhailov, V.V., Levanova, G.F., Garagulya, A.D., Gorshkova, N.M., et al., 1998. Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description. International Journal of Systematic Bacteriology 48, 247-256.

Jaffrés, E., Lalanne, V., Macé, S., Cornet, J., Cardinal, M., Sérot, T., Dousset, X. and Joffraud, J.J., 2011. Sensory characteristics of spoilage and volatile compounds associated with bacteria isolated from cooked and peeled tropical shrimps using SPME-GC-MS analysis. International Journal of Food Microbiology 147, 195-202.

Jaffrés, E., Sohier, D., Leroi, F., Pilet, M.F., Provost, H., Joffraud, J.J., et al., 2009. Study of the bacterial ecosystem in tropical cooked and peeled shrimps using a polyphasic approach. International Journal of Food Microbiology 131, 20-29.

Jay, J.M., 1986. Modern food microbiology, 3th ed. Van Nostrand Reinhold Company, New York.

Jeppesen, V.F. and Huss, H.H., 1993. Characteristics and antagonistic activity of lactic acid bacteria isolated from chilled fish products. International Journal of Food Microbiology18, 305-320.

Joffraud, J.J., Cardinal, M., Cornet, J., Chasles, J.S., Leon, S., Gigout, F. and Leroi, F., 2006. Effect of bacterial interactions on the spoilage of cold-smoked salmon. International Journal of Food Microbiology112, 51-61.

Joffraud, J. J. & Leroi, F. 2000. Spoilage and safety of cold-smoked fish. Final report of european contract FAIR-PL-95-1207.

Joffraud, J.J., Leroi, F., Roy, C. and Berdague, J.L., 2001. Characterisation of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon. International Journal of Food Microbiology, 66, 175-184.

Jonker, K.M., Roessink, G.L., Hamerlinck, E.M. and Schout, L.J., 1992. Chemisch en microbiologisch onderzoek van Noordzee-, Noor(d)se en tropische garnalen. De Ware(n)-Chemicus 22, 193-207.

Jonker, K.M., Vliegenthart, J.S. and de Boer, E., 2000. Microbiologische gesteldheid van garnalen. De Ware(n)-Chemicus 30, 177-180.

Jonsdottir, R., Olafsdottir, G., Chanie, E. and Haugen, J.E., 2008. Volatile compounds suitable for rapid detection as quality indicators of cold smoked salmon (*Salmo salar*). Food Chemistry 109, 184-195.

Jorgensen, B.R., Gibson, D.M. and Huss, H.H., 1988. Microbiological quality and shelf life prediction of chilled fish. International Journal of Food Microbiology6, 295-307.

Jorgensen, B.R. and Huss, H.H., 1989. Growth and activity of *Shewanella putrefaciens* isolated from spoiling fish. International Journal of Food Microbiology9, 51-62.

Jorgensen, L.V., Huss, H.H. and Dalgaard, P., 2000. The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon. Journal of Applied Microbiology 89, 920-934.

Jung, S. Y., Lee, M. H., Oh, T. K., Park, Y. H., Yoon, J. H., & Park, Y. H. 2005. *Psychrobacter cibarius* sp nov., isolated from jeotgal, a traditional Korean fermented seafood. International Journal of Systematic and Evolutionary Microbiology, 55, 577-582.

Juni, E. and Heym, G.A., 1986. *Psychrobacter immobilis* gen. nov., sp. nov. genospecies composed of Gram-negative, aerobic, oxidase-positive coccobacilli. International Journal of Systematic Bacteriology 36, 388-391.

Kampfer, P., Lodders, N., Vaneechoutte, M. and Wauters, G., 2009. Transfer of *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* to the genus *Chryseobacterium* as *Chryseobacterium antarcticum* comb. nov., *Chryseobacterium jeonii* comb. nov and *Chryseobacterium marinum* comb. nov. International Journal of Systematic and Evolutionary Microbiology 59, 2238-2240.

Karl, H. and Meyer, C., 2007. Effect of early gutting on shelf life of saithe (*Pollachius virens*), haddock (*Melanogrammus aeglefinus*) and plaice (*Pleuronectes platessa*) stored in ice. Journal fur Verbraucherschutz und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety 2, 130-137.

Kaspar, C.W. and Tamplin, M.L., 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Applied and Environmental Microbiology 59, 2425-2429.

Kelly, R.H. and Yancey, P.H., 1999. High contents of trimethylamine oxide correlating with depth in deep-sea teleost fishes, skates, and decapod crustaceans. Biological Bulletin 196, 18-25.

Kiessling, A., Stien, L.H., Torslett, V., Suontarna, J. and Slinde, E., 2006 .Effect of pre- and post-mortem temperature on rigor in Atlantic salmon muscle as measured by four different techniques. Aquaculture 259, 390-402.

Kim, S.-J., Park, S.-J., Oh, Y.-S., Lee, S.-A., Shin, K.-S., Roh, D.-H. and Rhee, S.-K., 2011. *Shewanella artica* sp. nov., an iron reducing bacterium isolated from Arctic marine sediment. International Journal of Systematic and Evolutionary Microbiology.

Koutsoumanis, K. and Nychas, G.J., 2000. Application of a systematic experimental procedure to develop a microbiological model for rapid fish shelf life predictions. International Journal of Food Microbiology, 60, 171-184.

Koutsoumanis, K. and Nychas, G.J.E., 1999. Chemical and sensory changes associated with microbiological flora of Mediterranean boque (*Boops boops*) stored aerobically at 0, 3, 7, and 10 degrees C. Applied and Environmental Microbiology, 65, 698-706.

Kudaka, J., Horii, T., Tamanaha, K., Itokazu, K., Nakamura, M., Taira, K. et al. 2010. Evaluation of the petrifilm aerobic count plate for enumeration of aerobic marine bacteria from seawater and *Caulerpa lentillifera*. Journal of Food Protection, 73, 1529-1532.

Kyrana, V.R. and Lougovois, V.P., 2002. Sensory, chemical and microbiological assessment of farm-raised European sea bass (*Dicentrarchus labrax*) stored in melting ice. International Journal of Food Science and Technology 37, 319-328.

La Duc, M.T., Satomi, M., Agata, N. and Venkateswaran, K., 2004. *Gyr*B as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. Journal of Microbiological Methods 56, 383-394.

Lalitha, K.V., Sonaji, E.R., Manju, S., Jose, L., Gopal, T.K.S. and Ravisankar, C.N., 2005. Microbiological and biochemical changes in pearl spot (*Etroplus suratensis* Bloch) stored under modified atmospheres. Journal of Applied Microbiology 99, 1222-1228.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H. et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics, 23, 2947-2948.

Larsen, P., Heldbo, J., Jespersen, C. M. and Nielsen, J. 1992. Development of a method for quality assessment of fish for human consumption based on sensory evaluation. In Quality assurance in the fish industry ed. Huss,H.H., Jakobsen,M. and Liston,J. pp. 351-358. Elsevier.

Lauzon, H.L., Magnusson, H., Sveinsdottir, K., Gudjonsdottir, M. and Martinsdottir, E., 2009. Effect of brining, modified atmosphere packaging, and superchilling on the shelf life of cod (*Gadus morhua*) loins. Journal of Food Science 74, M258-M267.

Leroi, F., 2010. Occurrence and role of lactic acid bacteria in seafood products. Food Microbiology 27, 698-709.

Lindberg, A.M., Ljungh, A., Ahrne, S., Lofdahl, S. and Molin, G., 1998. *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurised milk or cream and the presence of toxin encoding genes. International Journal of Food Microbiology, 39, 11-17.

Liston, J., 1980. Microbiology in fishery science. In: Connell, J.J. (ed.), Advances in fish science and technology. Fishing News Book Ltd. Farnham, Surrey, England, pp. 138-157.

Love, R. M. 1970. The chemical biology of fishes. London: Academic Press.

Ma, L.Y., Deng, J.C., Ahmed, E.M. and Adamo, J.P., 1983. Canned Shrimp Texture as a Function of Its Heat History. Journal of Food Science 48, 360-363.

Makarios-Laham, I.K. and Lee, T.-C., 1993. Protein hydrolysis and quality deterioration of refrigerated and frozen seafood due to obligately psychrophilic bacteria. Journal of Food Science 58, 310-313.

Malle, P., Valle, M., Eb, P. and Tailliez, R., 1998. Optimization of culture conditions for enumeration of H_2S bacteria in the flesh of seafish. Journal of Rapid Methods and Automation in Microbiology 6, 129-141.

Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Herman, L., De Block, J. and Heyndrickx, M., 2009. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi,* predominant milk spoilers isolated from Belgian raw milk samples. Environmental Microbiology 11, 467-482.

Marklinder, I.M., Lindblad, M.,. Eriksson, L.M, Finnson, A.M. and Lindqvist, R., 2004. Home storage temperatures and consumer handling of refrigerated foods in Sweden. Journal of Food Protection 67, 2570-2577.

Mauguin, S. and Novel, G., 1994 Characterization of lactic acid bacteria isolated from seafood. Journal of Applied Bacteriology 76, 616-625.

McMeekin, T. A. 1982. Microbiological spoilage of chicken breast muscle. In Developments in Food Microbiology ed. Davies,R. pp. 1-40. London: Applied Science Publishers.

Mejlholm, O., Boknaes, N. and Dalgaard, P., 2005. Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging. Journal of Applied Microbiology 99, 66-76.

Mendes, R., Huidobro, A. and Caballero, E.L., 2002a. Indole levels in deepwater pink shrimp (*Parapenaeus longirostris*) from the Portuguese coast. Effects of temperature abuse. European Food Research and Technology 214, 125-130.

Meziti, A., Ramette, A., Mente, E. and Kormas, K.A., 2010. Temporal shifts of the Norway lobster (*Nephrops norvegicus*) gut bacterial communities. FEMS Microbiology Ecology 74, 472-484.

Michaud, L., Di, M.G., Bruni, V. and Lo, G.A., 2007. Biodegradative potential and characterization of psychrotolerant polychlorinated biphenyl-degrading marine bacteria isolated from a coastal station in the Terra Nova Bay (Ross Sea, Antarctica). Marine Pollution Bulletin 54, 1754-1761

Miwa, S., Kamaishi, T., Matsuyama, T., Hayashi, T. and Naganobu, M., 2008. Histopathology of Antarctic krill, *Euphausia superba*, bearing black spots. Journal of Invertebrate Pathology 98, 280-286.

Molin, G. and Stenström, I.-M., 1984. Effect of temperature on the microbiological flora of herring fillets stored in air or carbon dioxide. Journal of Applied Bacteriology 56, 275-282.

Moore, E.R.B., Tindal, B.J., Dos Santos, V.A.P.M., Pieper, D.H, Ramos, J.-L. and Palleroni, N.J., 2006. Nonmedical: *Pseudomonas*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E. (eds.), The Prokaryotes - A handbook on the biology of bacteria. 3 ed. vol. 6. Springer, New York, pp. 646-703.

Moyer, R.H., Southcott, B.A., Baker, E.G. and Tarr, H.L.A., 1959. Keeping quality of Pacific coast dogfish. Journal of the Fisheries Research Board of Canada 16, 791-794.

Múgica, B., Barros-Velázquez, J., Miranda, J. M., & Aubourg, S. P. 2008. Evaluation of a slurry ice system for the commercialization of ray (*Raja clavata*): Effects on spoilage mechanisms directly affecting quality loss and shelf-life. LWT - Food Science and Technology, 974-981.

Mukundan, M.K., Antony, P.D. and Nair, M.R., 1986. A review on autolysis in fish. Fisheries Research 4, 259-269.

Muyzer, G., 1999. PCR-DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology, 2, 317-322.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G., 1993. Profiling of complex microbiological populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695-700.

Nemecek-Marshall, M., Wojciechowski, C., Wagner, W.P. and Fall, R., 1999. Acetone formation in the vibrio family: a new pathway for bacterial leucine catabolism. Journal of Bacteriology 181, 7493-7499.

Niamnuy, C., Devahastin, S. and Soponronnarit, S., 2007. Quality changes of shrimp during boiling in salt solution. Journal of Food Science, 72, 289-297.

Nicholls, D. G. and Ferguson, S. J. 2002. Bioenergetics 3. London: Academic Press.

Nilsson, L., Gram, L. and Huss, H.H., 1999. Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competitive lactic acid bacteria flora. Journal of Food Protection 62, 336-342.

Noller, H.F., 1984. Structure of ribosomal RNA. Annual Review of Biochemistry 53, 119-162.

Nordic Committee of Food Analysis. NMKL 184. Aerobic count and specific spoilage organisms in fish and fish products. 184:2006

Noseda, B., Dewulf, J., Goethals, J., Ragaert, P., Van Bree, I., Pauwels, D., Van Langenhove, H. and Devlieghere, F., 2010. Effect of Food Matrix and pH on the Volatilization of Bases (TVB) in Packed North Atlantic Gray Shrimp (*Crangon crangon*): Volatile Bases in MAP Fishery Products. Journal of Agricultural and Food Chemistry 58, 11864-11869.

Nychas, G. J. E., Marshall, D. L. and Sofos, J. N. 2007. Meat, Poultry, and Seafood. In Food Microbiology: Fundamentals and Frontiers ed. Doyle,M.P. and Beuchat,L.R. pp. 105-140. Washington: ASM Press.

Ocano-Higuera, V.M., Maeda-Martinez, A.N., Marquez-Rios, E., Canizales-Rodriguez, D.F., Castillo-Yanez, F.J., Ruiz-Bustos, E., Graciano-Verdugo, A.Z. and Plascencia-Jatomea, M., 2011. Freshness assessment of ray fish stored in ice by biochemical, chemical and physical methods. Food Chemistry 125, 49-54.

Olafsdottir, G., Chanie, E., Westad, F., Jonsdottir, R., Thalmann, C.R., Bazzo, S., Labreche, S., Marcq, P., Lundby, F. and Haugen, J.E., 2005a. Prediction of microbiological and sensory quality of cold smoked Atlantic salmon (*Salmo salar*) by electronic nose. Journal of Food Science 70, S563-S574.

Olafsdottir, G., Jonsdottir, R., Lauzon, H.L., Luten, J. and Kristbergsson, K., 2005b. Characterization of volatile compounds in chilled cod (*Gadus morhua*) fillets by gas chromatography and detection of quality indicators by an electronic nose. Journal of Agricultural and Food Chemistry 53, 10140-10147.

Olafsdottir, G., Lauzon, H.L., Martinsdottir, E. and Kristbergsson, K., 2006a. Influence of storage temperature on microbiological spoilage characteristics of haddock fillets (*Melanogrammus aeglefinus*) evaluated by multivariate quality prediction. International Journal of Food Microbiology 111, 112-125.

Olafsdottir, G., Lauzon, H.L., Martinsdottir, E., Oehlenschlager, J. and Kristbergsson, K., 2006b. Evaluation of shelf life of superchilled cod (*Gadus morhua*) fillets and the influence of temperature fluctuations during storage on microbiological and chemical quality indicators. Journal of Food Science 71, S97-S109.

Olafsdottir, G., Martinsdottir, E., Oehlenschlager, J., Dalgaard, P., Jensen, B., Undeland, I., Mackie, I.M., Henehan, G., Nielsen, J. and Nilsen, H., 1997. Methods to evaluate fish freshness in research and industry. Trends in Food Science & Technology 8, 258-265.

Olivares, A., Dryahina, K., Navarro, J.L., Flores, M., Smith, D. and Spanel, P., 2010. Selected ion flow tube-mass spectrometry for absolute quantification of aroma compounds in the headspace of dry fermented sausages. Analytical Chemistry 82, 5819-5829.

Olofsson, T.C., Ahrne, S. and Molin, G., 2007. The bacterial flora of vacuum-packed cold-smoked salmon stored at 7 degrees C, identified by direct 16S rRNA gene analysis and pure culture technique. Journal of Applied Microbiology 103, 109-119.

Oxley, A.P., Shipton, W., Owens, L. and McKay, D., 2002. Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. Journal of Applied Microbiology 93, 214-223.

Ozogul, F. and Ozogul, Y., 2007. The ability of biogenic amines and ammonia production by single bacterial cultures. European Food Research and Technology 225, 385-394.

Paarup, T., Sanchez, J.A., Moral, A., Christensen, H., Bisgaard, M. and Gram, L., 2002. Sensory, chemical and bacteriological changes during storage of iced squid (*Todaropsis eblanae*). Journal of Applied Microbiology 92, 941-950.

Palleroni, N.J., Kunisawa, J.R., Contopoulou, R. and Doudoroff, M., 1973. Nucleic acid homologies in the genus *Pseudomonas*. International Journal of Systematic Bacteriology 23, 333-339.

Palys, T., Nakamura, L.K. and Cohan, F.M., 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. International Journal of Systematic Bacteriology 47, 1145-1156.

Papadopoulos, V., Chouliara, I., Badeka, A., Savvaidis, I.N. and Kontominas, M.G., 2003. Effect of gutting on microbiological, chemical, and sensory properties of aquacultured sea bass (*Dicentrarchus labrax*) stored in ice. Food Microbiology 20, 411-420.

Pastoriza, L. and Sampedro, G., 1993. Prospects for the utilization of ray as a canned product. Nippon Suisan Gakkaishi 59, 1539-1544.

Peixoto, R.S., Coutinho, H.L.D., Rumjanek, N.G., Macrae, A. and Rosado, A.S., 2002. Use of *rpo*B and 16S rRNA genes to analyse bacterial diversity of a tropical soil using PCR and PCR-DGGE. Letters in Applied Microbiology 35, 316-320.

Pittard, B.T., Later, D.W., Lee, M.L. and Freeman, L.R., 1982. Identification of volatile organic-compounds produced by fluorescent pseudomonads on chicken breast muscle. Applied and Environmental Microbiology 43, 1504-1506.

Polet, H., Delanghe, F., and Verschoore, R. 2005. On electrical fishing for brown shrimp (*Crangon crangon*) II. Sea trials. Fisheries Research 72,13-27.

Poli, B.M., Messini, A., Parisi, G., Scappini, F., Vigiani, V., Giorgi, G. and Vincenzini, M., 2006. Sensory, physical, chemical and microbiological changes in European sea bass (*Dicentrarchus labrax*) fillets packed under modified atmosphere/air or prepared from whole fish stored in ice. International Journal of Food Science and Technology 41, 444-454.

Prapaiwong, N., Wallace, R.K. and Arias, C.R., 2009. Bacterial loads and microbiological composition in high pressure treated oysters during storage. International Journal of Food Microbiology 131, 145-150.

Ravesi, E.M., Licciardello, J.J., Tuhkunen, B.E. and Lundstrom, R.C., 1985. The effect of handling or processing treatments on storage characteristics of fresh spiny dogfish, *Squalus acanthias*. Marine Fisheries Review 47, 48-67.

Renouf, V., Claisse, O., Miot-Sertier, C. and Lonvaud-Funel, A., 2006. Lactic acid bacteria evolution during winemaking: Use of *rpo*B gene as a target for PCR-PCR-DGGE analysis. Food Microbiology 23, 136-145.

Reynisson, E., Lauzon, H.L., Magnusson, H., Hreggvidsson, G.O. and Marteinsson, V.T., 2008. Rapid quantitative monitoring method for the fish spoilage bacteria *Pseudomonas*. Journal of Environmental Monitoring 10, 1357-1362.

Reynisson, E., Lauzon, H.L., Magnusson, H., Jonsdottir, R., Olafsdottir, G., Marteinsson, V., et al., 2009. Bacterial composition and succession during storage of North-Atlantic cod (*Gadus morhua*) at superchilled temperatures. BMC Microbiology 9, 250.

Ringo, E. and Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177-203.

Rodriguez, O., Losada, V., Aubourg, S.P. and Barros-Velazquez, J., 2005. Sensory, microbiological and chemical effects of a slurry ice system (*Trachurus trachurus*). Journal of the Science of Food and Agriculture 85, 235-242.

Rodriguez-Calleja, J.M., Patterson, M.F., arcia-Lopez, I., Santos, J.A., and Garcia-Lopez,M.L., 2005. Incidence, radioresistance, and behavior of *Psychrobacter* spp. in rabbit meat. Journal of Food Protection 68, 543.

Romanenko, L. A., Lysenko, A. M., Rohde, M., Mikhailov, V. V., & Stackebrandt, E. 2004. *Psychrobacter maritimus* sp nov and *Psychrobacter arenosus* sp nov., isolated from coastal sea ice and sediments of the Sea of Japan. International Journal of Systematic and Evolutionary Microbiology, 54, 1741-1745.

Romero, J., Gonzalez, N. and Espejo, R.T., 2002. Marine *Pseudoalteromonas* sp. composes most of the bacterial population developed in oysters (*Tiostrea chilensis*) spoiled during storage. Journal of Food Science 67, 2300-2303.

Rudi, K., Maugesten, T., Hannevik, S.E. and Nissen, H., 2004. Explorative multivariate analyses of 16S rRNA gene data from microbiological communities in modified-atmosphere-packed salmon and coalfish. Applied and Environmental Microbiology 70, 5010-5018.

Saito, T., Arai, K. and Matsuyoshi, M., 1959. A new method for estimating the freshness of fish. Bulletin of the Japanese Society of Scientific Fisheries 24, 749-750.

Satomi, M., Vogel, B. F., Venkateswaran, K., & Gram, L. 2007. Description of *Shewanella glacialipiscicola* sp nov and *Shewanella algidipiscicola* sp nov., isolated from marine fish of the Danish Baltic Sea, and proposal that *Shewanella affinis* is a later heterotypic synonym of *Shewanella colwelliana*. International Journal of Systematic and Evolutionary Microbiology, 57, 347-352.

Schmitt, R.E. and Schmidtlorenz, W., 1992. Degradation of amino-acids and protein-changes during microbiological spoilage of chilled unpacked and packed chicken carcasses. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie 25, 11-20.

Schubring, R., 2002. Influence of freezing/thawing and frozen storage on the texture and colour of brown shrimp (*Crangon crangon*). Archiv fur Lebensmittelhygiene 53, 34-36.

Schubring, R. and Meyer, C., 1999. Qualitätsvergleich zwischen maschinen- und handgeschälten Nordseegarnelen. Inf. Fischwirtsch. Fischereiforsch. 46, 36-44.

Sen, D.P. 2005. Advances in fish processing technology. pp. 823. New Delhi, Allied publishers Pvt.Ltd.

Shewan, J. M. 1961. The microbiology of sea-water fish. In Fish as food, vol 1, Production, Biochemistry and Microbiology ed. Borgstrom,G. pp. 487-560. New York: Academic Press.

Shewan, J. M. 1962. The bacteriology of fresh and spoiling fish and some related chemical changes. In Recent advances in food science ed. Hawthorn,J. and Muil Leitch,J. pp. 167-193.

Shewan, J. M. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. pp. 51-66.

Shewan, J.M. and Georgala, D.L., 1957. The Effect of Spoilage and Handling on the Bacterial Flora of Fish. Proceedings of the Nutrition Society 16, 161-163.

Shewan, J.M., Hobbs, G. and Hodgkiss, W., 1960. A determinative scheme for the identification of certain genera of gram-negative bacteria, with special reference to the *Pseudomonadaceae*. Journal of Applied Bacteriology 23, 379-390.

Shewan, J.M. and Murray, C.K., 1979. The microbiological spoilage of fish with special reference to the role of psychrofiles. Society for Applied Bacteriology Technical Series 13, 117-136.

Skjervold, P.O., Fjaera, S.O., Ostby, P.B. and Einen, O., 2001. Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 192, 265-280.

Smith, D. and Spanel, P., 2005. Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis. Mass Spectrometry Reviews 24, 661-700.

Stackebrandt, E. and Ebers, J., 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today november 2006, 152-155.

Stansby, M. E. 1962. Proximate composition of fish. In Fish in nutrition ed. Heen, E. and Kreuzer, R. pp. 55-60. London: Fishing News Books Ltd.

Stenström, I.-M. and Molin, G., 1990. Classification of the spoilage flora of fish, with special reference to *Shewanella putrefaciens*. Journal of Applied Bacteriology 68, 601-618.

Stohr, V., Joffraud, J.J., Cardinal, M. and Leroi, F., 2001. Spoilage potential and sensory profile associated with bacteria isolated from cold-smoked salmon. Food Research International 34, 797-806.

Stroud, D. G. 2001. Rigor in fish - The effect on quality. FAO - TORRY ADVISORY NOTE No. 36

Surette, M.E., Gill, T.A. and Leblanc, P.J., 1988. Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. Journal of Agricultural and Food Chemistry 36, 19-22.

Tacao, M., Moura, A., Alves, A., Henriques, I., Saavedra, M.J. and Correia, A., 2005. Evaluation of 16S rDNA- and gyrB-PCR-DGGE for typing members of the genus *Aeromonas*. Fems Microbiology Letters 246, 11-18.

Tayeb, L.A., Ageron, E., Grimont, F. and Grimont, P.A.D., 2005. Molecular phylogeny of the genus *Pseudomonas* based on *rpo*B sequences and application for the identification of isolates. Research in Microbiology 156, 763-773.

Towner, K. 2006. The Genus *Acinetobacter*. In M.Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), The Prokaryotes - A Handbook on the Biology of Bacteria (3 ed., pp. 746-758). New York: Springer.

Triqui, R., 2006. Sensory and flavor profiles as a means of assessing freshness of hake (*Merluccius merluccius*) during ice storage. European Food Research and Technology 222, 41-47.

Tryfinopoulou, P., Drosinos, E.H. and Nychas, G.J., 2001. Performance of *Pseudomonas* CFC-selective medium in the fish storage ecosystems. Journal of Microbiological Methods 47, 243-247.

Tryfinopoulou, P., Tsakalidou, E. and Nychas, G.J., 2002. Characterization of *Pseudomonas* spp. associated with spoilage of gilt-head sea bream stored under various conditions. Applied and Environmental Microbiology 68, 65-72.

Tsironi, T., Dermesonlouoglou, ., Giannakourou, M. and Taoukis, P., 2009. Shelf life modelling of frozen shrimp at variable temperature conditions. LWT - Food Science and Technology 42, 664-671.

Tzikas, Z., Amvrosiadis, I., Soultos, N. and georgakis, Sp., 2007. Seasonal variation in the chemical composition and microbiological condition of Mediterranean horse mackerel (*Trachurus mediterraneus*) muscle from the North Aegean Sea (Greece). Food control 18, 251-257.

Ursing, J., 1986. Similarities of Genome Deoxyribonucleic Acids of *Pseudomonas* Strains Isolated from Meat. Current Microbiology 13, 7-10.

Uyttendaele, M., Jacxsens, L., De Loy-Hendrickx, A., Devlieghere, F. and Debevere, J. 2010. Microbiological guide values and legal criteria. Ghent: Ghent University.

Valdimarsson, G., Einarsson, H., Gudbjornsdottir, B. and Magnusson, H., 1998. Microbiological quality of Icelandic cooked-peeled shrimp (*Pandalus borealis*). International Journal of Food Microbiology45, 157-161.

Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A. and Soulas, G., 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. FEMS Microbiology Ecology 24, 279-285.

Vallé, M., Eb, P., Tailliez, R. and Malle, P., 1998. Optimization of the enumeration of total aerobic bacterial flora in the flesh of seafish. Journal of Rapid Methods and Automation in Microbiology 6, 29-42.

Van De Peer, Y. & De Wachter, R. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Bioinformatics, 10, 569-570.

Van Spreekens, K.J.A., 1974. The suitability of a Long and Hammer's medium for the enumeration of more fastidious bacteria from fresh fishery products. Archiv für Lebensmittelhygiene 25, 213-219.

Van Spreekens, K.J.A., 1977. Characterisation of some fish and shrimp spoiling bacteria. Antonie Van Leeuwenhoek 43, 283-303.

Van Spreekens, K.J.A. and de Man, T.A., 1970. Enkele microbiologische aspecten van gekookte garnalen. Voedingsmiddelentechnology 1, 290-292.

van Wintzingerode, F., Göbel, U.B. and Stackebrandt, E., 1997. Determination of microbiological diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews 21, 213-229.

Varlet, V. and Fernandez, X., 2010. Review. Sulfur-containing volatile compounds in seafood: occurrence, odorant properties and mechanisms of formation. Food Science and Technology International 16, 463-503.

Versalovic, J., Koeuth, T. and Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19, 6823-6831.

Vogel, B.F., Venkateswaran, K., Satomi, M. and Gram, L., 2005. Identification of *Shewanella baltica* as the most important H_2S-producing species during iced storage of Danish marine Fish. Applied and Environmental Microbiology 71, 6689-6697.

Vyncke, W., 1978a. Determination of ammonia in dressed thornback ray (*Raja clavata* L.) as a quality test. Journal of Food Technology 13, 37-44.

Vyncke, W., 1978b. Influence of sodium tripolyphosphate and citric acid on shelf life of thornback ray (*Raja clavata* L). Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung 166, 284-286.

Wang, T., Sveinsdottir, K., Magnusson, H. and Martinsdottir, E., 2008. Combined application of modified atmosphere packaging and superchilled storage to extend the shelf life of fresh cod (*Gadus morhua*) loins. Journal of Food Science 73, S11-S19.

Wiedemeijer, J. and Pateer, P.M., 1984. Onderzoek naar de microbiologische gesteldheid van niet bevroren gekookte gepelde garnalen uit de detailhandel. De Ware(n)-Chemicus 14, 1-6.

Wilson, B., Danilowicz, B.S. and Meijer, W.G., 2008. The diversity of bacterial communities associated with Atlantic cod *Gadus morhua*. Microbiology Ecology 55, 425-434.

WIV. 2006. De Belgische Voedselconsumptiepeiling 1 – (2004). Devriese, S., Huybrechts, I., Moreau, M. and Van Oyen, H. Afdeling Epidemiologie, 2006; Brussel Wetenschappelijk Instituut Volksgezondheid, Depotnummer : D/2006/2505/17, IPH/EPI REPORTS N° 2006 – 016. http://www.iph.fgov.be/epidemio/epien/index5.htm

Woese, C.R., 1987. Bacterial evolution. Microbiological Reviews 51, 221-271.

Yamamoto, S. and Harayama, S., 1995. PCR amplification and direct sequencing of *gyr*b genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Applied and Environmental Microbiology 61, 1104-1109.

Yoon, J. H., Kang, K. H., & Park, Y. H. 2003. *Psychrobacter jeotgali* sp nov., isolated from jeotgal, a traditional Korean fermented seafood. International Journal of Systematic and Evolutionary Microbiology, 53, 449-454.

Yumoto, I., Hirota, K., Sogabe, Y., Nodasaka, Y., Yokota, Y. and Hoshino, T., 2003. *Psychrobacter okhotskensis* sp nov., a lipase-producing facultative psychrophile isolated from the coast of the Okhotsk Sea. International Journal of Systematic and Evolutionary Microbiology 53, 1985-1989.

Yumoto, I., Kusano, T., Shingyo, T., Nodasaka, Y., Matsuyama, H. and Okuyama, H., 2001. Assignment of *Pseudomonas* sp strain E-3 to *Pseudomonas psychrophila* sp nov., a new facultatively psychrophilic bacterium. Extremophiles 5, 343-349.

Zaballos, M., Lopez-Lopez, A., Ovreas, L., Bartual, S.G., D'Auria, G., Alba, J.C., Legault, B., Pushker, R., Daae, F.L. and Rodriguez-Valera, F., 2006. Comparison of prokaryotic diversity at offshore oceanic locations reveals a different microbiota in the Mediterranean Sea. FEMS Microbiology Ecology 56, 389-405.

Zapatka, F.A. and Bartolomeo, B., 1973. Microbiological evaluation of cold-water shrimp (*Pandalus borealis*). Applied Microbiology 25, 858-861.

Zeng, Q.Z., Thorarinsdottir, K.A. and Olafsdottir, G., 2005. Quality changes of shrimp (*Pandalus borealis*) stored under different cooling conditions. Journal of Food Science 70, S459-S466.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W., 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7, 203-214.

SUMMARY

SAMENVATTING

Summary

After an outline of the objectives of this thesis (**chapter 1**), a profound literature overview of seafood spoilage and quality analysis techniques in general is given in **chapter 2**. Several aspects of spoilage are described from *rigor mortis*, autolysis and microbiological changes to biochemical changes under aerobic conditions. Also the different ways of assessing the quality of seafood by sensorial, microbiological, biochemical or physical evaluation in the industry is briefly described.

Since seafood spoilage is mainly caused by microbiological growth and activity it's important to detect a profound view of the microbiota present on seafood. Therefore, the first part (**Part I**) of this thesis consists of a profound investigation and evaluation of the traditional detection techniques.

It is known that total count analysis on various general media gave different results concerning the total aerobic psychrotolerant counts. To this end, the microbiological community on eleven fish species during storage on ice was analysed through several plating methods and identified and compared through molecular techniques. In **chapter 3**, several methods, traditional and molecular, confirmed that on plate count agar the enumeration of the microbiota was much lower and revealed that many microorganisms were not detected. However, a generalization as described in literature that total counts on plate count agar are approximately one log lower than on marine media could not be made. The difference in counts on various media depended on the fish species and therefore the present microbiota. Partial 16S rRNA gene and *gyrB* gene sequencing identified members of the genera *Photobacterium*, *Shewanella*, *Vibrio*, *Aliivibrio*, *Pseudoalteromonas*, *Psychrobacter*, *Brochothrix*, *Flavobacterium*, *Acinetobacter*, *Pseudomonas* and *Janthinobacterium* as not growing on one of the media studied. This study therefore provides further evidence that plate count agar, an official ISO method, is not the most suitable growth medium for fish analysis and fish spoilage quality. Especially since some well-known SSOs such as *Photobacterium phosphoreum*, *Shewanella baltica* and *Pseudomonas fluorescens* are unable to grow on it. Marine media such as Long and Hammer agar and marine agar seemed to obtain the best quantitative and qualitative results for fish quality analysis during this study, however also these media have restrictions.

The second part of this thesis, divided in part II and part III, consists of the implementation of the optimized techniques in order to identify and characterise the dominant microbiota of two

typical Belgian fishery products, namely brown shrimp (*Crangon crangon*) and ray (*Raja* sp.). Since previous research revealed that not the total number of microbiota on fish is responsible for fish spoilage, but rather only a small fraction of the microorganisms, the "specific spoilage organisms" or SSOs it's important that those microorganisms are detected and identified in order to be able to determine the seafood quality and remaining shelf life. These SSOs are seafood specific and are responsible for the production of volatile organic compounds (VOCs) associated with spoilage.

In **Part II** the dominant microbiota of brown shrimp (**chapter 4**) and their spoilage potential (**chapter 5**) is studied.

In **chapter 4**, the dominant microbiota of brown shrimp (*Crangon crangon*) without preservatives were identified during storage under different conditions. Therefore freshly caught shrimp were caught and processed on board under the best possible hygienic conditions. These shrimp were unpeeled and manually (sterile) peeled and stored on ice and at 7.5°C until microbiologically spoiled. The results were then compared with industrially processed shrimp without preservatives. However the microbiota differed somewhat during storage and among the various storage conditions; members of the genera *Psychrobacter* and *Pseudoalteromonas* were found to dominate the microbiota of all shrimp samples regardless of processing procedures or storage conditions. Identification via partial and nearly full 16S rRNA gene sequencing resulted for both genera in species complexes. *Gyr*B gene sequencing however, was able to identify more isolates to species level. *Psychrobacter immobilis*, *Psychrobacter cibarius*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas elyakovii* and *Pseudoalteromonas paragorgicola* were found to dominate the microbiota of brown shrimp during storage. Also species from the genera *Planococcus*, *Exiguobacterium*, *Carnobacterium*, *Pseudomonas*, *Chryseobacterium* and *Staphylococcus* were detected in lower numbers during storage of brown shrimp.

Also a culture-dependent and culture-independent PCR-DGGE analysis was performed resulting in different results in band patterns between both methods. Both methods are therefore interesting to compare in order to accurately identify the microbiota and bacterial population shifts on seafood during storage.

Chapter 5 deals with the spoilage potential of the isolates detected in chapter 4. The spoilage potential of *Psychrobacter cibarius*, *Psychrobacter maritimus*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas paragorgicola* and *Pseudoalteromonas nigrifaciens* was determined and

quantified in this study based on the presence of VOCs. These isolates were inoculated as pure cultures on heat-sterilised shrimp. The inoculated samples were stored at 4°C and the production of VOCs by the pure strains on the shrimp matrix was identified via gas chromatography coupled to mass spectrometry (GC-MS). VOC production was quantified daily by selected ion flow tube mass spectrometry (SIFT-MS) until the bacterial count exceeded 10^8-10^9 cfu/g. The sensory profile of *Psychrobacter* species revealed very low spoilage potential as measured by the production of VOCs. However, these species may nevertheless contribute to spoilage; based on the API ZYM test, a way to observe the species' enzymatic capacity to contribute to spoilage by degrading lipids, amino acids and proteins, *Pseudoalteromonas* as well as *Psychrobacter* species might enhance spoilage by breaking down lipids and hydrolysing amino acids and proteins. *Pseudoalteromonas* species, especially *Pseudoalteromonas elyakovii* and *Pseudoalteromonas nigrifaciens*, do have a high spoilage potential, however, and might be responsible for some off-odours produced during spoilage of brown shrimp. These isolates produced significant amounts of volatile compounds such as sulphides, acetone, ammonia, ethanol, etc., which are all involved in seafood spoilage.

Part III describes the dominant microbiota of ray and their spoilage potential.

The dominant microbiota of ray, an elasmobranch fish known for its fast deterioration due to the conversion of ureum to ammonia, was identified during storage on ice in **chapter 6**. Isolates grown on various media were identified by partial 16S rRNA, *gyr*B and *rpo*B gene sequencing. Microbiological shifts were observed during storage from the initial microbiota (e.g. *Arthrobacter*, *Flavobacterium, Pseudomonas*) to a dominance of members of the genera *Pseudomonas* and *Psychrobacter* at the end of storage time. 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*. Different storage conditions e.g. late gutting, resulted in the presence of other species in large numbers such as *Pseudoalteromonas* spp., *Shewanella putrefaciens* and *Staphylococcus* spp.

Subsequently, also 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 as performed for brown shrimp in chapter 5. 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 ureum to ammonia. The isolates were inoculated as pure cultures on gamma-sterilised ray, were stored at 4°C and the VOC production was determined daily by GC-MS and SIFT-MS analyses. The obtained 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.

In the last chapter the **general conclusions, recommendations and perspectives** were discussed. The limitations of the techniques were debated and possible solutions were provided for further research. Also the SSOs of brown shrimp and ray were discussed with an emphasis on how further research is necessary and how those SSOs can be eliminated in order to ameliorate shelf life.

Samenvatting

Na het beschrijven van de doelstellingen van de thesis (**Hoofdstuk 1**), wordt in **hoofdstuk 2** een grondige literatuurstudie weergegeven over het bederf van visserijproducten en de huidige technieken, gebruikt voor de kwaliteitsanalyses van visserijproducten. Hierbij worden de verschillende aspecten omtrent bederf zoals *rigor mortis*, autolytische en microbiologische veranderingen, maar ook biochemische veranderingen onder aerobe condities beschreven. Tevens worden de verschillende methodes die tot op heden gebruikt worden om de kwaliteit van visserijproducten zowel sensorisch, microbiologisch, biochemisch als fysisch te evalueren, kort aangehaald.

Aangezien bederf van visserijproducten voornamelijk gebeurt door bacteriële groei en activiteiten, is het belangrijk om een zo volledig mogelijk overzicht te genereren van alle microbiota op het te bestuderen visserijproduct. Daarom bestaat het eerste deel van deze thesis (**Deel I**) uit een diepgaande studie en evaluatie van de traditionele detectiemethoden die tegenwoordig gebruikt worden in het onderzoek naar de microbiologische kwaliteit van vis en visserijproducten.

In de viswereld is algemeen geweten dat verschillende algemene groeimedia andere psychrotrofisch kiemgetal resultaten zullen weergeven van hetzelfde staal. Daarom werd op elf vissoorten met verschillende typische kenmerken de aanwezige microbiologische gemeenschappen gedurende bewaring op ijs geanalyseerd aan de hand van verschillende conventionele plaattechnieken. Vervolgens werden deze resultaten vergeleken met de resultaten verkregen via moleculaire technieken en werden de isolaten genetisch geïdentificeerd. In **hoofdstuk 3** werd bevestigd dat plate count agar een veel lager totaal kiemgetal weergeeft dan andere (mariene) groeimedia. Tevens bleek dat verschillende micro-organismen niet op deze bodem kunnen groeien. Maar het verschil in totaal kiemgetal verschilde naargelang de vissoort en bijgevolg ook met de aanwezige microbiota. Via gedeeltelijke 16S rRNA gen en *gyr*B gen sequentiebepaling werden volgende genera teruggevonden: *Photobacterium*, *Shewanella*, *Vibrio*, *Aliivibrio*, *Pseudoalteromonas*, *Psychrobacter*, *Brochothrix*, *Flavobacterium*, *Acinetobacter*, *Pseudomonas* en *Janthinobacterium*. Hierdoor bewijst dit onderzoek dat het gebruik van plate count agar, een officiële ISO methode, eigenlijk niet geschikt is voor microbiologisch onderzoek of kwaliteitsbepaling op vis- en visserijproducten van mariene oorsprong. Dit wordt benadrukt doordat verschillende specifieke bederforganismen zoals *Photobacterium phosphoreum*,

Shewanella baltica en *Pseudomonas fluorescens* niet op plate count agar kunnen groeien. Hoewel mariene media zoals Long en Hammer medium en mariene agar zowel kwalitatief als kwantitatief het beste leken in deze studie, hebben ook deze groeimedia beperkingen.

Het tweede deel van deze thesis, opgesplitst in 2 aparte delen (Deel II en Deel III), bestaat uit het identificeren en karakteriseren van de dominante microbiota van twee typische Belgische visserijproducten, namelijk de grijze garnaal (*Crangon crangon*) en rog (*Raja* sp.). Onderzoek heeft aangetoond dat slechts een kleine fractie van de aanwezige microbiota op vis, namelijk de specifieke bederforganismen of SBO's verantwoordelijk zijn voor het bederf van visserijproducten. Om de kwaliteit en de resterende bewaartijd van visserijproducten te bepalen is het belangrijk dat deze SBO's gedetecteerd en geïdentificeerd worden. Deze SBO's zijn niet alleen verantwoordelijk voor de productie van vluchtige organische componenten die geassocieerd zijn met bederf, ze zijn ook specifiek voor een bepaald visserijproduct onder specifieke bewaaromstandigheden.

In **deel II** werd de dominante microbiota van grijze garnalen (**Hoofdstuk 4**) en hun bederfpotentieel (**Hoofdstuk 5**) bestudeerd.

In **hoofdstuk 4**, gebeurde de identificatie van de dominante microbiota van grijze garnalen zonder bewaarmiddelen onder verschillende bewaarcondities. Hiervoor werden verse garnalen gevangen en aan boord verwerkt onder de meest steriele condities. Deze garnalen werden zowel gepeld (manueel steriel) als ongepeld bewaard op ijs en bij 7.5°C tot ze (microbiologisch) bedorven waren. De resultaten werden dan vergeleken met industrieel verwerkte garnalen zonder bewaarmiddelen. Hoewel er soms kleine verschillen optraden in de microbiota tijdens bewaring of onder verschillende condities, werden voornamelijk *Psychrobacter* en *Pseudoalteromonas* soorten gevonden die de microbiota van de grijze garnaal domineerden onafhankelijk van de verwerkings- of bewaarcondities. Identificatie van deze isolaten via het 16S rRNA gen resulteerde voor beide genera in soortcomplexen. Via hun *gyr*B sequentie werden verschillende isolaten tot op soortsniveau geïdentificeerd, namelijk *Psychrobacter immobilis*, *Psychrobacter cibarius*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas elyakovii* en *Pseudoalteromonas paragorgicola*. Hiernaast werden er ook een aantal genera (*Planococcus*, *Exiguobacterium*, *Carnobacterium*, *Pseudomonas*, *Chryseobacterium* en *Staphylococcus*) teruggevonden in lagere aantallen. Tussen cultuurafhankelijke en cultuuronafhankelijke PCR-DGGE technieken op dezelfde stalen werden eveneens verschillen in bandenpatronen teruggevonden. Het wordt dan ook

aanbevolen om beide technieken naast elkaar te gebruiken om de microbiota grondig te identificeren en eventuele populatieverschuivingen te detecteren tijdens bewaring.

In **hoofdstuk 5** wordt het bederfpotentieel van de verkregen isolaten uit hoofdstuk 4 (*Psychrobacter cibarius*, *Psychrobacter maritimus*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas paragorgicola* en *Pseudoalteromonas nigrifaciens*) bepaald en gekwantificeerd aan de hand de productie van vluchtige organische componenten (VOC's) bepaald en gekwantificeerd. Hiervoor werden deze isolaten als pure cultuur geïnoculeerd op hitte-gesteriliseerde garnalen. Deze geïnoculeerde stalen werden bewaard bij 4°C en de productie van VOC's werd geïdentificeerd via GC-MS en dagelijks gekwantificeerd via SIFT-MS tot een totaal kiemgetal van 10^8-10^9 werd bereikt. Aan de hand van het sensorisch profiel op basis van VOC productie werd het bederfpotentieel van *Psychrobacter* als relatief laag beschouwd, wat echter niet betekent dat deze soorten niet kunnen bijdragen aan bederf. Op basis van de API ZYM test, waarmee men bepaalde enzymatische capaciteiten van een isolaat kan bepalen, bleek dat zowel *Pseudoalteromonas* als *Psychrobacter* soorten in staat waren om vetten af te breken en aminozuren en eiwitten te hydrolyseren. *Pseudoalteromonas* soorten, en dan voornamelijk *Pseudoalteromonas elyakovii* en *Pseudoalteromonas nigrifaciens* kunnen door hun productie van sulfiden, aceton, ammoniak, ethanol en dergelijke mogelijks wel bijdragen tot de productie van de bederfgeur bij grijze garnalen.

Deel III van deze thesis beschrijft de dominante microbiota van rog en hun bederfpotentieel.

Rog is een kraakbeenvis welke gekenmerkt is door snelle productie van een afwijkende ammoniak geur na vangst. In **hoofdstuk 6** werd de dominante microbiota van deze vissoort geïdentificeerd. Hiervoor werden isolaten afgepikt van verschillende groeimedia en geïdentificeerd via hun partiele 16S rRNA, *gyr*B en *rpo*B gen sequentie. Tijdens bewaring op ijs werden populatieverschuivingen opgemerkt van de meer initiële populatie (bv. *Arthrobacter*, *Flavobacterium, Pseudomonas*) naar de dominante populatie met *Pseudomonas* en *Psychrobacter* soorten op het einde van bewaring op ijs. Via *rpo*B (*Pseudomonas* spp.) en *gyr*B sequentie analyse konden de meeste isolaten geïdentificeerd worden als *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas psychrophila*, *Psychrobacter cibarius*, *Psychrobacter cryohalolentis*, *Psychrobacter glacincola* en *Psychrobacter immobilis*. Afhankelijk van de bewaarcondities, bv. laat gutten, konden ook andere soorten zoals *Pseudoalteromonas* spp., *Shewanella putrefaciens* en *Staphylococcus* spp. in hoge aantallen geïdentificeerd worden.

Na identificatie volgde de karakterisatie van het bederfpotentieel van een aantal isolaten (*Flavobacterium tegetincola*, *Pseudomonas fluorescens*, *Pseudomonas psychrophila*, *Psychrobacter cibarius*, *Psychrobacter cryohalolentis* en *Shewanella frigidimarina*) zoals in hoofdstuk 5 uitgevoerd werd voor grijze garnalen. Naast de VOC productie werden ook een API ZYM en urease test uitgevoerd om hun enzymatische bederf capaciteit te bepalen aan de hand van eventuele lipolytische of proteolytische activiteit, hydrolyse van aminozuren en eiwitten of de omzetting van ureum naar ammoniak. Zoals in hoofdstuk 5 werden pure isolaten geïnoculeerd op de steriele matrix, hier gamma gesteriliseerde rog, bewaard bij 4°C en werd dagelijks de VOC productie gekwantificeerd. Opnieuw werd gezien dat voornamelijk *Psychrobacter cibarius* en *Pseudomonas psychrophila* in staat waren om hogere concentraties aan VOCs te produceren en hierdoor mogelijks medeverantwoordelijk zijn voor de afwijkende geuren gedurende bederf van rog.

In het laatste hoofdstuk werden de **algemene conclusies, mogelijke aanbevelingen en perspectieven** voor verder onderzoek besproken. De beperkingen van de gebruikte technieken werden aangehaald gevolgd door mogelijke oplossingen voor verder onderzoek. Tevens werden de SBO's van garnaal en rog besproken met de nadruk op de noodzaak aan verder onderzoek om deze te elimineren en zo de bewaartijd mogelijks te verlengen.

CURRICULUM VITAE

Curriculum Vitae

Katrien Broekaert werd geboren op 18 februari 1983 te Zottegem. Nadat ze afgestudeerd aan het Onze Lieve Vrouw van Deinsbeke College te Zottegem in de richting Moderne Talen-Wetenschappen, studeerde ze verder aan de Universiteit Gent waar ze de richting Biologie aanvatte.

In het academiejaar 2005-2006 behaalde ze met grote onderscheiding het diploma Licentiaat in de Biologie. Vervolgens startte ze het daaropvolgend academiejaar de éénjarige ManaMa "Master in Molecular Biotechnology, option Medical" waarin ze in 2007 met grote onderscheiding afstudeerde.

Gebeten door de onderzoeksmicrobe startte ze in juli 2007 als bursaal op het Instituut voor landbouw en Visserij Onderzoek (ILVO) – Eenheid Technologie en Voeding, waar ze tot op heden tewerkgesteld is.

Katrien Broekaert is auteur van verscheidene wetenschappelijke publicaties en was actief deelnemer en spreker op nationale en internationale congressen.

Scientific activities

Papers in international journals

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F. and Vlaemynck, G. 2011. Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several growth media. Food Microbiology 28, 1162-1169.

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F. and Vlaemynck, G. 2011. Molecular identification of the microbiota of peeled and unpeeled brown shrimp (*Crangon crangon*) during storage on ice and at 7.5°C. Food Microbiology. Submitted for publication.

Broekaert, K., Noseda, B., Heyndrickx, M., Vlaemynck, G. and Devlieghere, F. 2011. Volatile compounds associated with *Psychrobacter* spp. and *Pseudoalteromonas* spp., the dominant microbiota of brown shrimp (*Crangon crangon*) during aerobic storage. International Journal of Food Microbiology. Submitted for publication.

Broekaert, K., Noseda, B., Heyndrickx, M., Vlaemynck, G. and Devlieghere, F. 2011. The spoilage microbiota of ray (*Raja* sp.) during ice storage under different conditions: molecular identification and characterisation of the spoilage potential. International Journal of Food Microbiology. In preparation.

Oral presentations on international symposia

Broekaert, K., Heyndrickx, M., Hoffman, S., Devlieghere, F., Herman, L., Vlaemynck, G. 2008. Microbiological spoilage of cooked and peeled brown shrimp (*Crangon crangon*). In 38th WEFTA annual meeting, 17-19 September 2008, Florence.

Broekaert, K., Heyndrickx, M., Devlieghere, F. and Vlaemynck, G. 2010. Molecular identification of fish spoilage microorganisms isolated from different general growth media. In 40th WEFTA annual meeting: Seafood: Getting most, serving best… 4 – 6 October 2010, Izmir.

Broekaert, K. and Devlieghere, F. 2011. Bederfflora van verse vis: identificatie en karakterisatie naar bederfpotentieel/Flores d'altération du poisson frais: de l'identification et la caractérisation au potentiel d'altération. In Sixteenth Conference on Food Microbiology, 22-23 September 2011, Brussels.

Abstracts of poster presentations on national and international conferences and symposia

Broekaert, K., Vlaemynck, G., Heyndrickx, M., Bekaert, K., Parmentier, K., Cooreman,* K. and Herman L. 2008. Quality of fish and fishery products, research at ILVO, Belgium. In The fifth SEAFOODplus conference, Abstract Book. 8-10 June, Copenhagen.

Broekaert, K., Heyndrickx, M., Hoffman, S., Devlieghere, F., Herman, L., Vlaemynck, G. 2009. Identification of spoilage micro-organisms of brown "Purus" shrimps (*Crangon crangon*). The Safe Consortium International Congress on Food Safety - Abstract Book (2th volume), pp 127., 27-29 April, Girona.

Broekaert, K., Heyndrickx, M., Herman, L., Devlieghere, F. and Vlaemynck, G. 2009. Evaluation of media in the detection of bacterial contaminants in fish. Book of abstracts of the 3th Joint Trans-Atlantic Fisheries Technology Conference. September 2009, pp. 85., 16-18 September, Copenhagen.

Broekaert, K., Heyndrickx, M., Hoffman, S., Devlieghere, F., Herman, L., Vlaemynck, G. 2009. Analysis of the microbiota of "Purus" shrimps (*Crangon crangon*) from catch to consumer. Proceedings of the Fourteenth Conference on Food Microbiology, pp. 89., 18-19 June, Luik.

Broekaert, K., Heyndrickx, M., Devlieghere, F., Herman, L. and Vlaemynck, G. 2009. Differences in the dominant microbiota present on various growth media applied in fish analysis. Analyzing complex microbiological communities and their host microbe interactions. Proceedings of the BSM symposium, pp. 26., 11 December, Brussels.

Broekaert, K., Van Coillie, E., Heyndrickx, M., Devlieghere, F. and Vlaemynck, G. 2010. Limitations of general growth media in fish analysis and research for SSOs. 22nd International ICFMH Symposium Food Micro August 2010 Final program and Abstract book, pp. 164., 30 August- 3 September, Copenhagen.

Broekaert, K., Heyndrickx, M., Devlieghere, F. and Vlaemynck, G. 2010. Limitations of general growth media in fish analysis and research for SSOs. EXCHANGE: Open innovation for feed, food & health. Where Industry and academia meet!, pp. 41., 28 September, Ghent.

Broekaert, K., Heyndrickx, M., Devlieghere, F., Herman, L. and Vlaemynck, G. (2011). The microbiota of common shrimps (*Crangon crangon*) from catch to consumer. VLIZ Young Scientists' Day, Book of abstracts, pp. 17., 25 February, Bruges.

Students

2009 – 2010 Pieter Siau (Professionele Bachelor Chemie afstudeerrichting Biochemie, KaHo Sint Lieven, Gent)
Identificatie van de dominante microbiota van rog tijdens bewaring

2010 – 2011 Adriaan Verhelle, (Stage, Master in de Bio-Ingenieurswetenschappen: cel- en genbiotechnologie, Universiteit Gent)
Microbiologische kwaliteitsparameters van visserij- en visproducten