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 = ± 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.

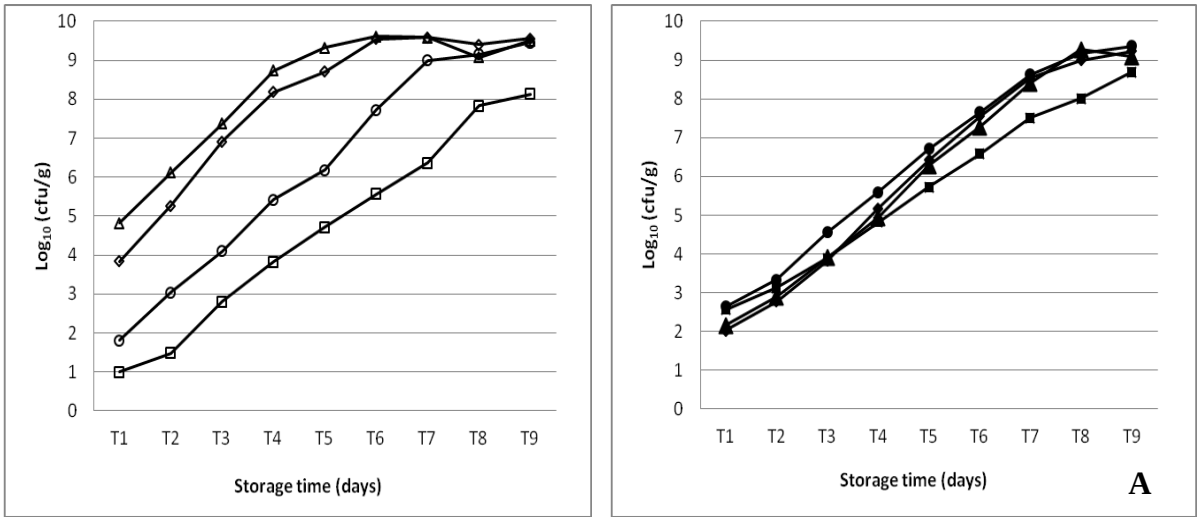

Figure 5.1. Growth of the bacterial isolates on chilled shrimp during storage at 4°C in air. The bacterial counts are given in $\log_{10}$ cfu/g. A: *Pseudoalteromonas* spp.: *Pseudoalteromonas nigrifaciens* (isolate 1G215 ◇), *Pseudoalteromonas elyakovii* (isolate 1G272 ○), *Pseudoalteromonas elyakovii* (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).

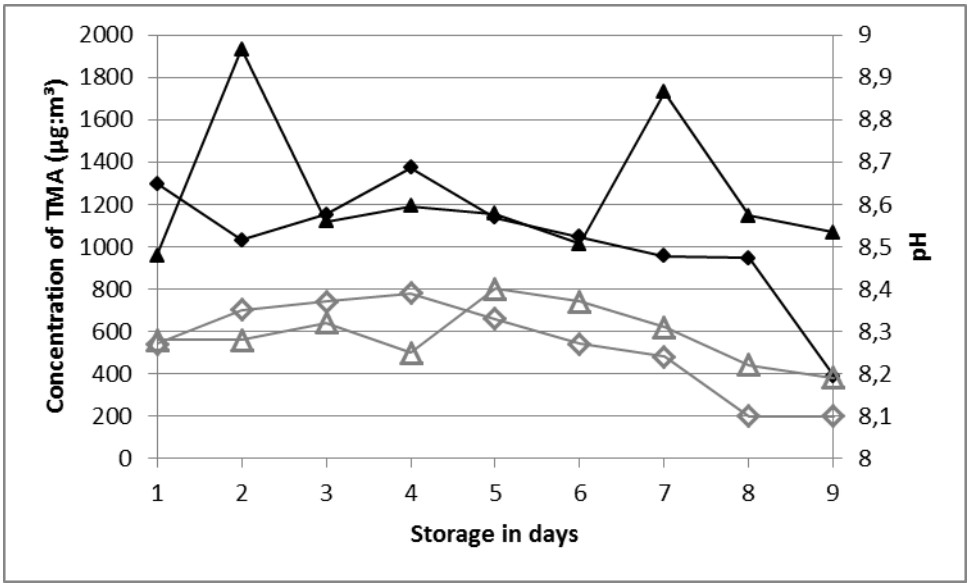

Fig. 5.2. pH and production of trimethylamine (TMA) (olfactory threshold by Devos et al., 1990: 5.89µg/m³) by *Psychrobacter cibarius* isolate 1G232 (◇ (pH) ◆ (TMA)) and *Psychrobacter maritimus* isolate 1G200 (△(pH) ▲ (TMA)), both in function of the storage time in days.

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.

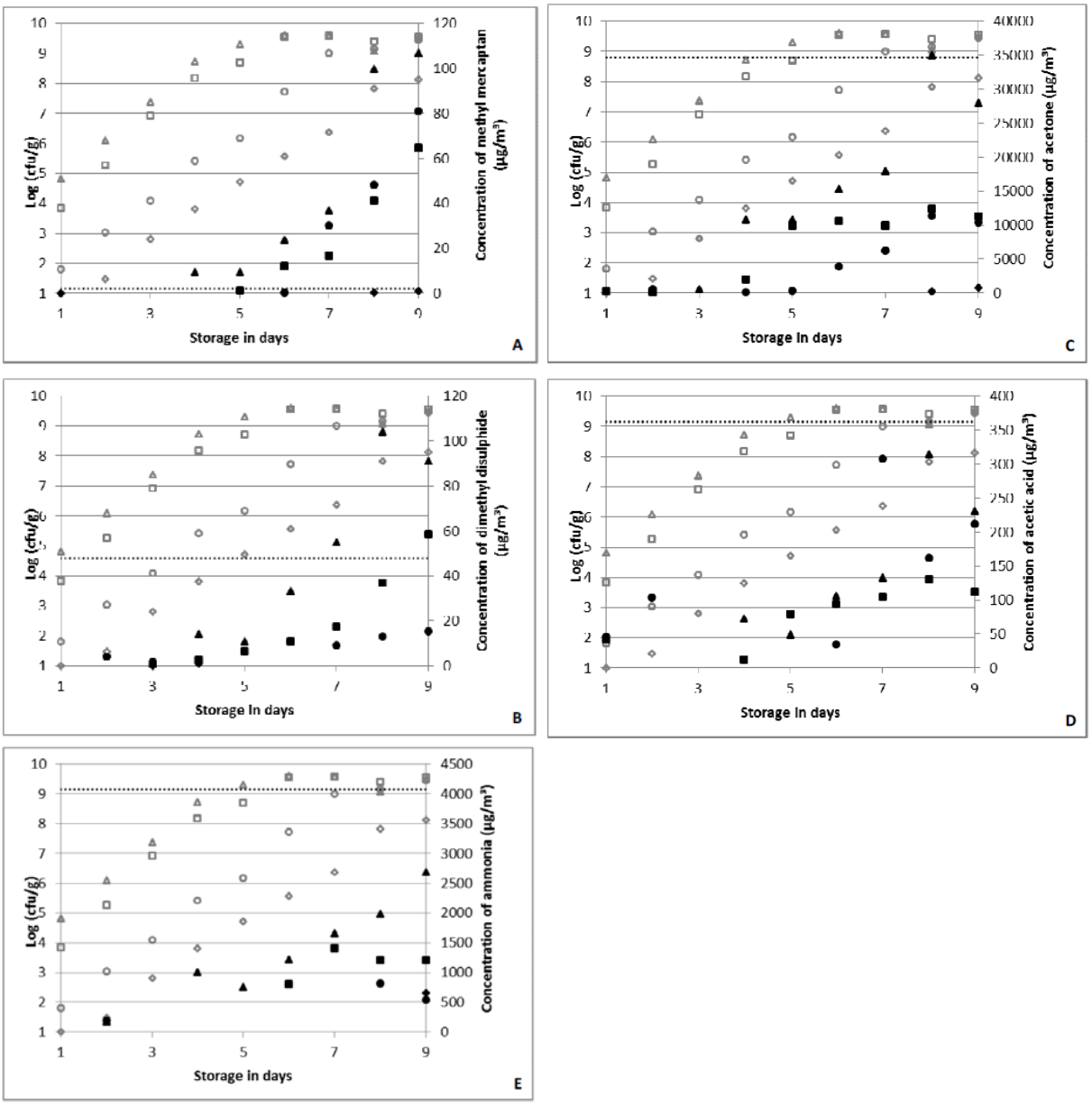

Fig. 5.3. Concentrations (in µg/m³) of (A) methyl mercaptan, (B) dimethyl disulphide, (C) acetone, (D) acetic acid and (E) ammonia produced by *Pseudoalteromonas elyakovii* (1G161 ▲), *Pseudoalteromonas elyakovii* (1G272 ●), *Pseudoalteromonas nigrifaciens* (1G215 ■) and *Pseudoalteromonas paragorgicola* (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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