MICROBIAL QUALITY OF FROZEN *PANGASIUS HYPOPHTHALMUS* AS INFLUENCED BY INDUSTRIAL PROCESSING IN VIETNAM

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

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Thesis title in Dutch:

De invloed van de industriële verwerking op de microbiologische kwaliteit van diepgevroren *Pangasius hypophthalmus* in Vietnam

Illustration on cover:

Pangasius hypophthalmus, processing plant, colony on medium plates, frozen *Pangasius* fillets

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Foreword

This PhD dissertation reflects a four-year journey of research in the Laboratory of Food Microbiology and Food Preservation of the Department of Food Safety and Food Quality of Ghent University. I would like to sincerely gratitude the people who contributed to this.

First of all, I would like to express my deep gratitude to my promotor, Prof. dr. ir. Frank Devlieghere. Not only did you give me the opportunity to work in your laboratory but you also provided a great motivation and orientation for me to reach the end of the journey. I am extremely grateful for your trust and also motivating me on my research. Especially, never being complained but I could know how you were patient during meetings! Thank you a lot for your kind contribution.

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To my (ex-) colleagues in the Laboratory of Food Microbiology and Food Preservation, I would like to thank you for all the support. I will remember the warm smile and enthusiastic guidance from everyone. I really do hope everyone remains a friendly icon of the Lab LFMFP as usual.

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To my colleagues at the Department of of Food Technology, Cantho University, I would like to thank you all for the great support during my samplings. I could not have done my PhD experiments if there had been no support.

I would like to thank all Vietnamese friends (especially to Chi Que, chi Loan, chi Ha, Phuong, Yen, Thien, Tung, Giang, Chi) and to all the friends who I have not mentioned yet. Without your spirit support, I would have not been able to cope with the difficulties here in Belgium. Indeed, I had a remarkable time "to enjoy" the happiness and sadness with all of you.

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This PhD dissertation is a result of teamwork. It is impossible to mention everyone who helped in and outside of the study. I fully appreciate your valuable contribution.

Thank You All

Anh Ngoc

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LIST OF ABBREVIATIONS AND SYMBOLS

-	Not determined
BAP	Best Aquaculture Practices
BRC	British Retail Consortium
CAC	Codex Alimentarius Commission
CCP	Critical Control Point
CFC	Cetrimide Fucidine Cephaloridine
CFU	Colony Forming Units
COD	Chemical Oxygen Demand
eV	Electronvolt
FS	Food Safety
FSMS	Food Safety Management System(s)
FSMS-DI	Food Safety Management System-Diagnostic Instrument
FSPI	Food Safety Performance Indicator
GHP	Good Hygiene Practices
GlobalGAP	Global Good Agricultural Practice
GMP	Good Manufacturing Practices
h	hour
HACCP	Hazard Analysis and Critical Control Points
ICMSF	International Commission on Microbiological Specifications for Foods
IFS	International Food Standard
ISO	International Organization for Standardization
LAB	Lactic Acid Bacteria
LFMFP	Laboratory of Food Microbiology and Food Preservation
LOD	Limit of detection
LOQ	Limit of quantification
MAP	Modified Atmosphere Packaging
min	minute
MRD	Maximum recovery diluent
mV	millivolts

NTU	Nephelometric Turbidity Unit
ORP	Oxidation reduction potential
PAA	Peracetic acid
PCR	Polymerase chain reaction
ppm	Part per million
PPS	Pepton Physiological Solution
PRP	Prerequisite Program
QA	Quality Assurance
rDNA	Ribosomal deoxyribonucleic acid
rep-PCR	Repetitive element sequence based polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	second
spp.	Species (plural)
THMs	Trihalomethanes
TMC	Total Mesophillic Count
TPC	Total Psychrotrophic Count
TSA	Trypton Soy Agar
TSB	Trypton Soy Broth
UV	Ultraviolet
VRBGA	Violet red bile glucose agar

OVERVIEW OF THE THESIS

Fish and fishery products are some of the most perishable food products as they are susceptible to rapid quality losses. Quality losses of fishery products are mainly the result of microbiological activity. The microbiota of fishery products varies and depends on the geographical location, the season, the temperature of water, the fish species, the slaughtering, treatment after slaughter, etc. Most of the quality loss is a result of the production of off-odors and -flavours by microbial contaminants. However, not all micro-organisms present on the product play an important role in the degradation of the product. Only a fraction of the microbial contaminants, termed specific spoilage organisms (Gram and Huss, 1996), participate in the degradation and loss of quality.

In addition to quality losses, producers also face microbial food safety-related challenges. As microbiological pathogens can contaminate products at all stages of the food chain (pre-harvest, handling, processing, distribution), the main goal of producers is to control pathogens in order to provide safe, wholesome, and acceptable food for consumers. However, this can be very challenging due to the variable performance of food safety management systems in practice. Specifically, insufficient sanitation, hygiene deficiencies and improper production practices can be the main reasons related to variability of food safety outputs. Therefore, there is a need for more knowledge on the (evolution of) microbiota of freshwater during processing and the influences of processing steps on the quality and safety of fishery products processed in countries such as Vietnam.

Pangasius hypophthalmus (Sauvage, 1878), a freshwater fish found in the Mekong Delta of Vietnam, plays an important role in aquaculture in Vietnam (Phan et al., 2009). In addition, the production of *Pangasius* products is still growing with annual production in excess of one million tons and export to over one hundred countries, indicating that *Pangasius* is highly and widely accepted for consumption (FAO, 2012; Phan et al., 2009; VASEP, 2013). Despite the booming export of *Pangasius* products from Vietnam, at the moment very little scientific information is available about microbiological quality of these products. Especially, the influence of processing on the microbial quality and safety of *Pangasius* is lacking. Most of the studies in literature on *Pangasius* have focused on farming practices and the environmental contamination of polluted water and soil (Andrieu et al., 2015; Anh et al., 2010;

Da et al., 2012). A few studies have reported the nutritional quality of Vietnamese *Pangasius* products such as the protein, lipid, ash, minerals, (non) essential amino acids content and so on (Karl et al., 2010; Orban et al., 2008; Ruiz-de-Cenzano et al., 2013; Szlinder-Richert et al., 2011). In contrast, the information of microbial quality as well as safety of *Pangasius* products marketed in local and international trade is very limited. Therefore, this thesis attempts to address these gaps, especially the study on microbial quality of *Pangasius hypophthalmus* as influenced by industrial processing. The overview of the thesis is as follows:

Chapter 1 reviews the scientific literature on the microbial quality and safety of fish during processing. The review begins with an analysis of the economic importance of *Pangasius* fish in Vietnam. Thereafter, the nutritional value is then described. Furthermore, the results of studies that have evaluated the microbiota of freshwater fish and *Pangasius* fish are discussed. Thereafter, the microbial spoilage and pathogens associated with *Pangasius* in particular are discussed. Lastly, the literature regarding the disinfection of wash water by chlorine and peracetic acid is summarized.

In **chapter 2**, the microbiota of *Pangasius* fish during processing in a large vs. small scale company in Vietnam and on processing lines using non-chlorinated and non-chlorinated water for washing was evaluated. The microbiota on samples collected throughout each processing line was identified by 16S rRNA gene sequencing. The microbial quality and ecology of frozen *Pangasius* products sold in Belgium is investigated in **chapter 3**.

The microbial quality and safety of *Pangasius* processed in Vietnam in the same companies evaluated in chapter 2 was evaluated by means of a microbiological assessment scheme (MAS) combined with questionnaires to determine the performance of the food safety management systems in place at those companies. The results of these studies are described in **chapter 4.**

In **chapter 5** a preliminary evaluation of the decontamination efficiency of washing *Pangasius* products in chlorinated water was performed as is currently done in a processing company in Vietnam. Thereafter, the decontamination of *Pangasius* fish and disinfection of the wash water by chlorine was studied on a laboratory scale. Next, in **chapter 6,** the continuous dosing of chlorine in the wash water was evaluated with focus on the decontamination of the fish and wash water in addition to the chemical safety.

The aim of **chapter 7** was to critically discuss the overall importance (implications) and potential applications of the results of this study and to offer perspectives for future studies to advance our understanding of the microbial safety and quality of fishery products.

Chapter 1 LITERATURE REVIEW: MICROBIAL QUALITY AND SAFETY OF *PANGASIUS* DURING PROCESSING

1.1 Overview of fish consumption

For many societies, fish and fishery products are a valuable source of high quality proteins, (polyunsaturated) fatty acids (i.e. omega-3 fatty acids), essential micronutrients and minerals. Fish can be consumed in various ways i.e. raw, cooked, grilled etc. or in various product forms such as salted, smoked, cured, canned and as component of ready-to-eat products. 40% of all fish intended for human consumption is marketed fresh (40%), whilst 32, 16 and 12% are processed into frozen, canned and cured products, respectively (Ababouch, 2006; Amagliani et al., 2012). The average per capita consumption of seafood and freshwater fish in various countries is listed in **Table 1.1.**

Table 1.1 Seafood and freshwater fish consumption by consumer worldwide in 2010 estimated by the Food and Agriculture Organization

Country	**Seafood (kg/person/year)**	**Freshwater fish (kg/person/year)**
World	18.7	6.7
European Union	22.9	3.8
USA	21.9	4.6
China	32.3	15.0
Japan	53.7	5.2
Belgium	25.2	6.0

Currently, Japan, the EU and the U.S. are the major importers of fish and fishery products (Ababouch, 2006). 46% of the total world demand of the fish and fishery products is satisfied by aquaculture products from Asia. China is the leading exporter of fish in Asia, followed by Thailand and Vietnam (FAO, 2010). Nowadays, fishery exports from Vietnam have increased by *ca.* 24-fold over the past two decades. Vietnam is now one of the top ten aquaculture exporters in the world. Of the various fish products produced in Vietnam, *Pangasius* species are rapidly becoming an increasingly important freshwater fish. The following discussion is therefore primarily focused on the production of *Pangasius* products.

1.2 The production of *Pangasius* products in Vietnam

1.2.1 Overview of farmed Pangasius *and its economic value*

Over the past two decades, the Mekong Delta has become a very important center for agricultural development in Vietnam. Important agricultural activities centered in the Mekong Delta include the production of rice, fruit and aquaculture. The Mekong Delta is located in the southern part of Vietnam and covers 12% of the total area of the country (331698 km²) (see **Figure 1.1**) and accounts for 72% of the country aquaculture production (Cuyvers and Van Binh, 2008). In recent years, the intensive development and the rapid expansion of *Pangasius hypoththalmus* production in the Mekong River Delta has resulted in a rapid increase in aquaculture in Vietnam (Phan et al., 2009). The total value of fish exports from the Mekong Delta regions accounts for 55% of Vietnam's total export income. Income from exports play an essential role in improving the income of local people and the rural economy (Cuyvers and Van Binh, 2008).

A B

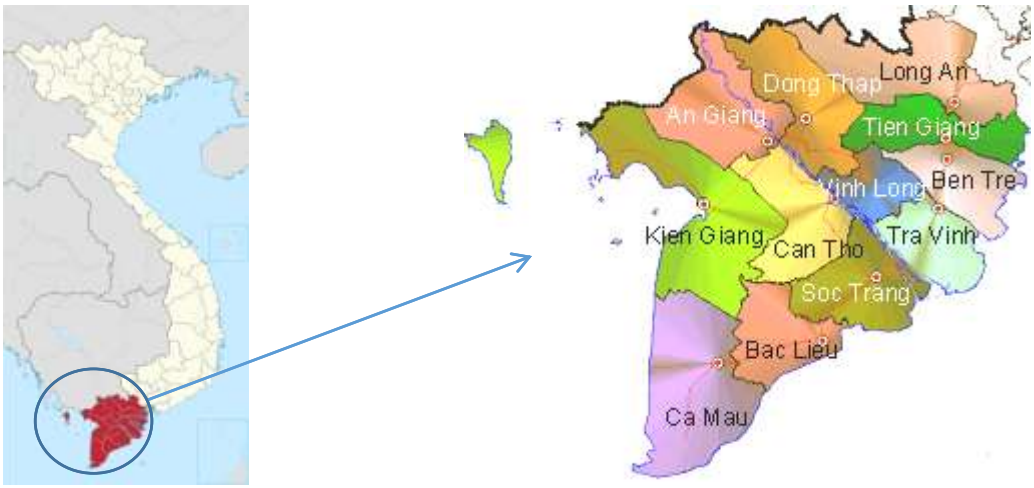


Figure 1.1 The map of Vietnam (A) and Mekong Delta (B)

Pangasius hypophthalmus is also referred to as *Pangasianodon hypophthalmus*. Common English names include sutchi catfish and striped catfish. It is known as Tra fish in the Mekong Delta regions. In the following discussion the term *Pangasius* will refer to Tra fish. *Pangasius* is naturally distributed in Mekong river and Chao Phraya river of Thailand. *Pangasius* was cultured in many countries (i.e. Vietnam, Thailand, Bangladesh, Myanmar, Cambodia, Lao, etc.), but currently production is predominant in the Mekong Delta, Vietnam (Nguyen, 2009). The *Pangasius* aquaculture in Vietnam was used to stock in ponds, mainly as supply for local

trade. Currently, *Pangasius* aquaculture is using much more intensified farming systems for international trade (Nguyen, 2009).

Figure 1.2 *Pangasius hypophthalmus* (VASEP, 2013)

The *Pangasius* (see **Figure 1.2**) are intensively farmed by stocking of hatchery-produced seed in ponds, floating cages or net-pen enclosures. Floating cages are made from steel or wood and consist of two parts: an under-water structure and a floating cage. In contrast, the net enclosure is a farming area in a river which is separated from the rest of the river by fences. The fences enable water to flow freely into the net enclosure thereby allowing for the water to be refreshed on a regular basis. The stock density varies according to the farming technique employed: ponds (30-40 fish/m^2), floating cages (100-150 fish/m^3) and net-pen enclosures (40-60 fish/m^2). A mature and commercial *Pangasius* fish, which weighs *ca*. 1.0 to 1.2 kg (the optimal weight for processing), can be harvested after 8 months of farming (Karl et al., 2010; VASEP, 2013). The average yield of *Pangasius* can reach 250-300 tons/ha in ponds, 0.1-0.12 tons/m^3 in floating cages, and 300-350 tons/ha in net-pen enclosures (VASEP, 2013). In 2011, the total farming area utilized for *Pangasius* in Vietnam was estimated to be *ca*. 5,509 ha in ten provinces located in the Mekong Delta (Dong Thap, An Giang, Can Tho, Ben Tre, Vinh Long, Tien Giang, Hau Giang, Soc Trang, Tra Vinh, and Kien Giang (see **Figure 1.3**) and two other provinces (Tay Ninh and Quang Nam) which are not located in the Mekong Delta.

Pangasius production reached *ca*. 1.43 million tons in 2011 according to Vietnam Association of Seafood Exporters and Producers (FAO, 2012; VASEP, 2013). In recent years, Vietnamese aquaculture production has increased steadily, reaching 1.6 million tons of *Pangasius* in 2012 (MARD, 2013; VASEP, 2013). According to Ministry of Agriculture and Rural Development, *Pangasius* farming will be expanded to 7,260 ha by 2020 (VASEP, 2014). As a result, *Pangasius* production should increase to *ca*. 2 million tons/year by 2020. Besides quantity,

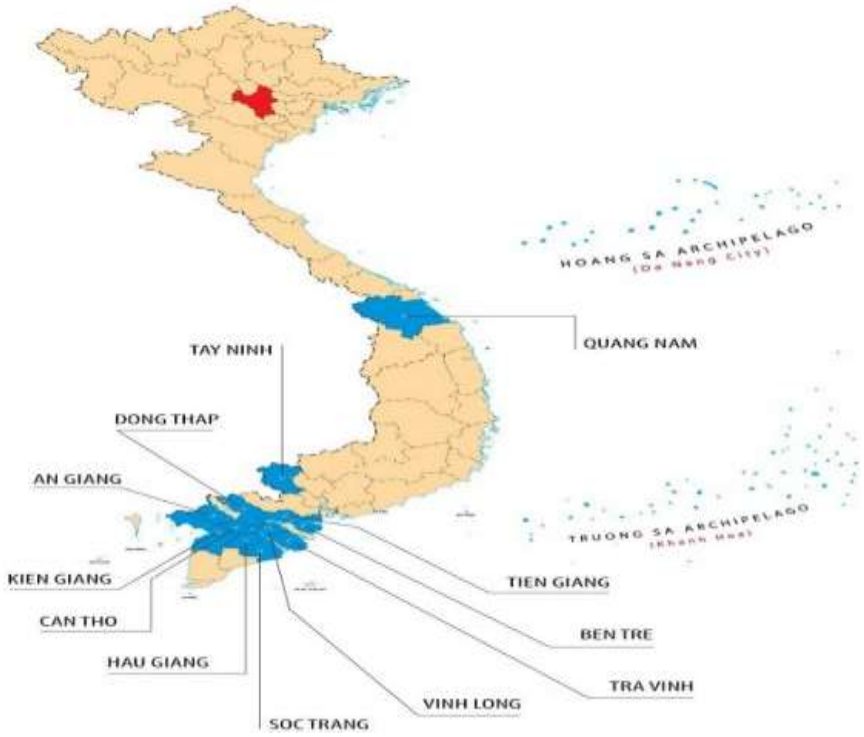


Figure 1.3 Farming *Pangasius* regions (highlighted in blue) in Vietnam (VASEP, 2013)

the quality of *Pangasius* raised has been controlled by the implementation of quality management systems for farming ponds such as SQF 1000 CM (Safety Quality Food for primary producers) as well as international standards i.e. Global Good Agricultural Practice (GlobalGAP) and Best Aquaculture Practices (BAP) (VASEP, 2013).

Recent statistics show that there has been an increase in the export of *Pangasius* products from 2005 to 2011. In the U.S trade, *Pangasius* products were ranked as the sixth most favorite fish in 2011 (Globefish, 2011). In the EU, the trade value from *Pangasius* products increased from $139 million in 2005 to $ 526 million in 2011. Global trade in *Pangasius* products has also increased gradually, from $328 million in 2005 to $1.856 million in 2011 (**Figure 1.4**). In similarity to the trend of the trade value, *Pangasius* production volumes exported from Vietnam to EU accounted for at least 20% of total *Pangasius* products exported throughout global trade (**Figure 1.5**). According to the Vietnam Association of Seafood Exporters and Producers (VASEP, 2014), the biggest markets during the first half of 2014 for Vietnamese *Pangasius* products were the European Union (21.0%) and the U.S. (18.4%) (**Figure 1.6**).

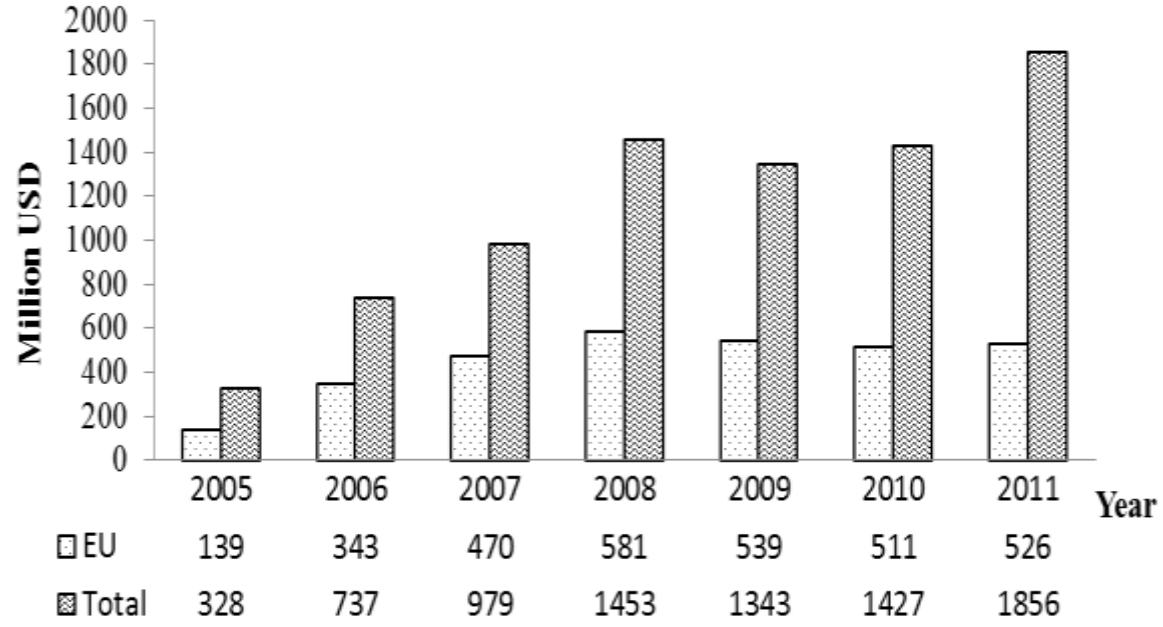


Figure 1.4 Economic value of *Pangasius* products exported from Vietnam between 2005 to 2011 (million USD) (Globefish, 2011)

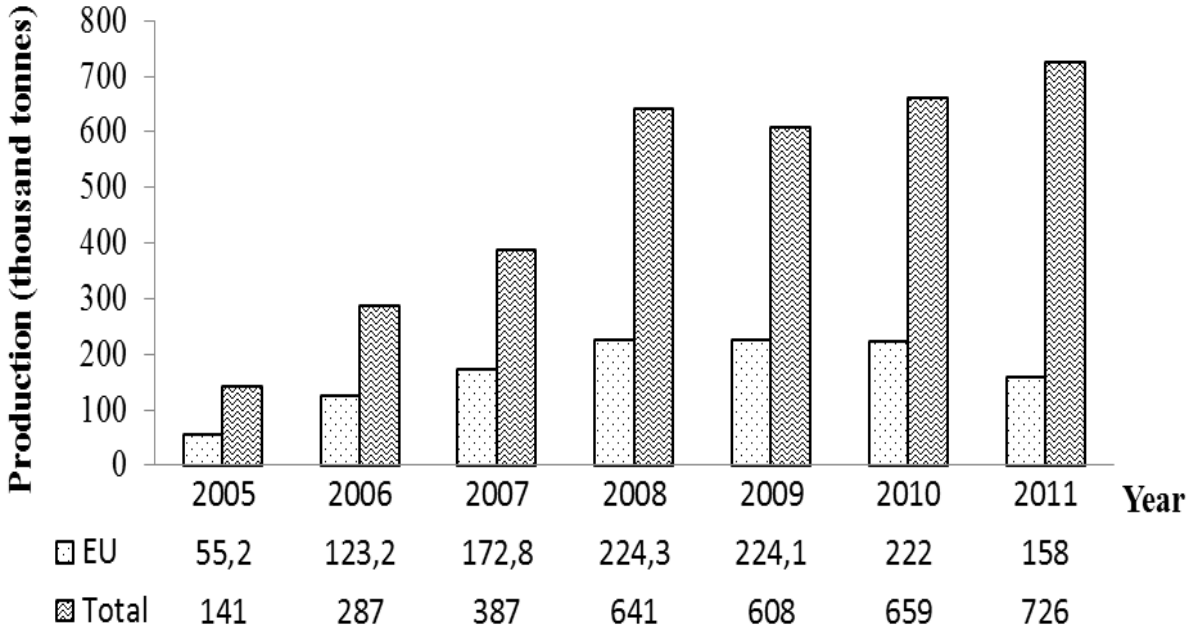


Figure 1.5 *Pangasius* production exported from Vietnam between 2005 and 2011 (thousand tons) (Globefish, 2011)

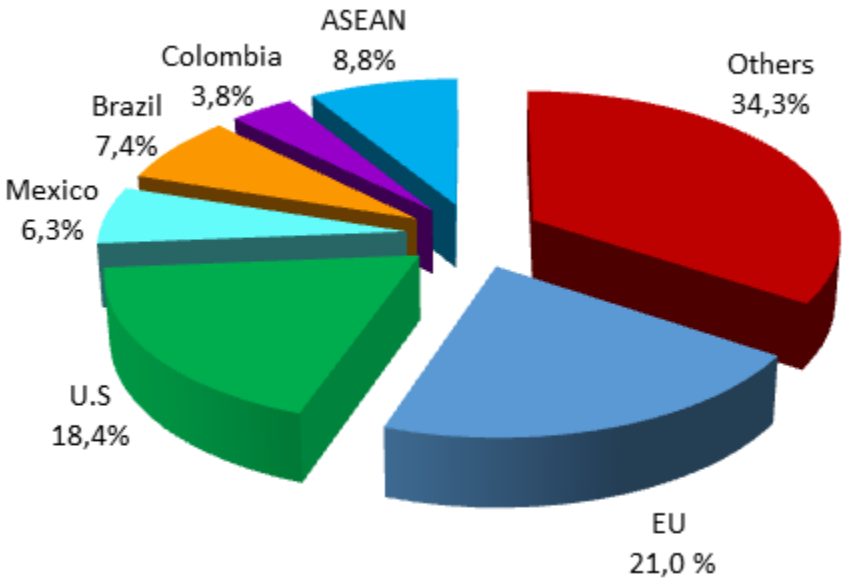


Figure 1.6 Export markets of Vietnamese *Pangasius* products (Jan-Jun, 2014) (VASEP, 2014)

1.2.2 Pangasius *products*

1.2.2.1 Processing flow chart

Fillets
(www.vietfish.org)

Steaks
(www.pangasius-vietnam.com)

Portions
(www.sohafood.com)

Added value products
(www.vinhhoan.com.vn)

Figure 1.7 Variety of *Pangasius* products for export

Pangasius is now largely accepted as an affordable substitute for cod and other white fish in Western countries (Phan et al., 2009). The a variety of *Pangasius* products marketed globally such as portions, steaks, fillets and its added value products (i.e. breaded, strips, ring *Pangasius*) (**Figure 1.7)** (VASEP, 2013). Furthermore, Orban et al. (2008) also pointed out that frozen and thawed fillets are the most popular forms sold in supermarkets and at fresh seafood markets in European countries. The European market commonly offers skinned and boneless frozen fillets (Karl et al., 2010).

In Vietnam, the processing of frozen *Pangasius* fillets including cutting of the gills, filleting, skinning, trimming, sorting, tumbling, cooling, packaging and freezing is shown in **Figure 1.8.** Frozen *Pangasius* is further processed as (re)fresh fillets i.e. thawed fillets and stored under modified atmospheres package. Therefore, not only frozen forms but also (re)fresh (thawed) fillets have become popular in Western markets. In this study, frozen *Pangasius* products processed in Vietnam and/or marketed in Belgium are investigated. The processing of frozen *Pangasius* fillet is described as follows:

- From farms to factories

Live *Pangasius* fish are normally transported from suppliers to the factory by boat. As the quality of final products depends on the stress level of the raw fish, the boats are usually equipped with an underwater hatch which enables the water in the cages to be refreshed with river water. Each boat can transport 10 to 12 tons of *Pangasius*. During transportation from farms to factories, factors such as fish density, water circulation and transport time will be taken into account to avoid exhausted (or dead) fish.

- Processing lines

Upon arrival at the company, bleeding is performed manually by cutting the main blood vessel (aorta) leading from the heart to the gills of *Pangasius*. The blood is pressed out *via* contractions of the heart of the fish. During bleeding (*ca.* 30 min.), the fish are dipped in a water bath. Thereafter, the *Pangasius* are filleted manually followed by washing in a continuous stream of water. The fillets are then skinned mechanically by passing them through rotating knives. Trimming is then performed, whereby the subcutaneous fat and red muscle on the surface of the fillets is scraped-off with a knife. A knife is also used to trim the edges of the fillets. The fillets are then sorted manually based on color into white, pink to red, and yellow groups. Every fillet is checked for (putative) parasites by placing them on a

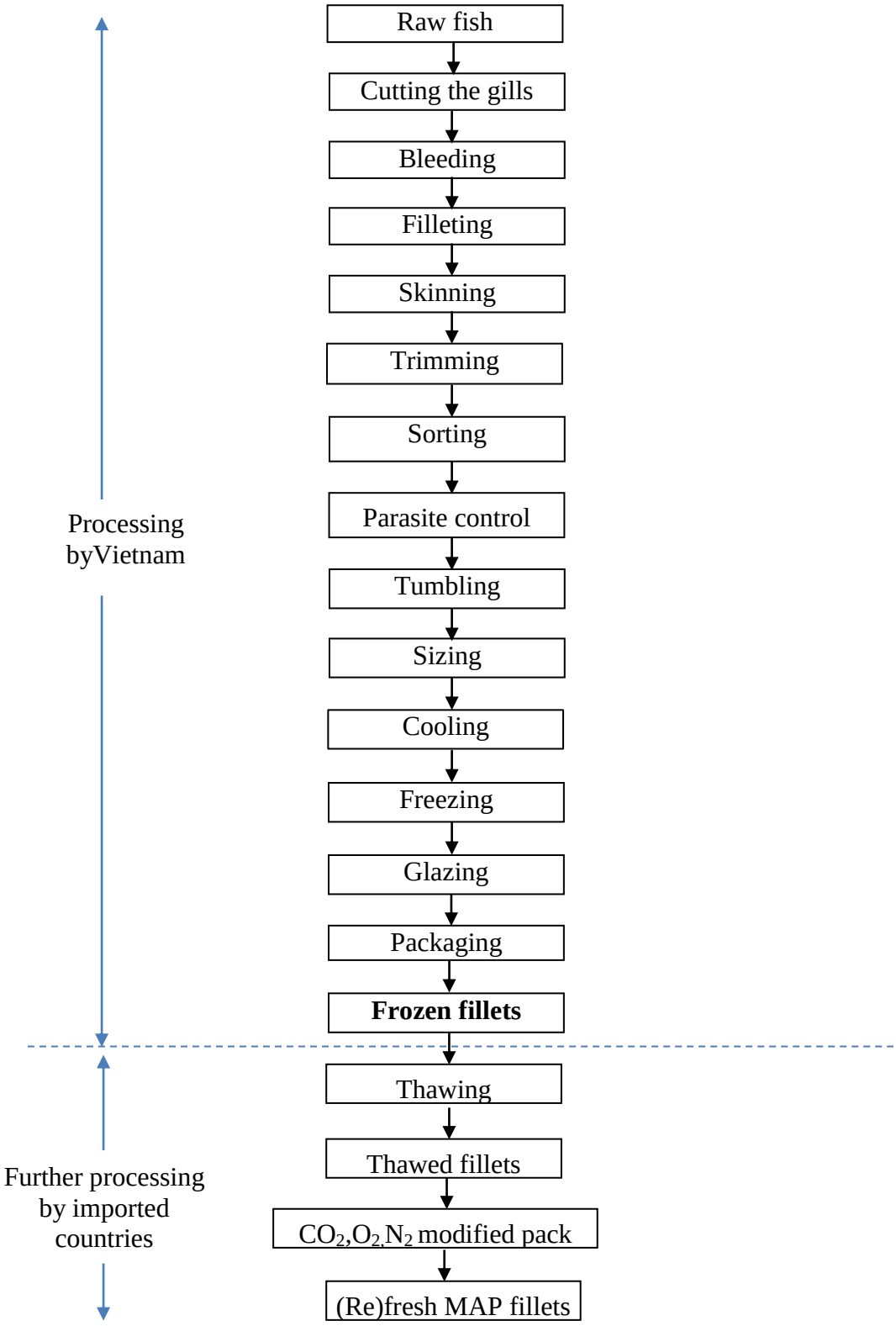


Figure 1.8 Flow chart for *Pangasius* products

translucent table illuminated from below. Depending on the processing line, water or chlorinated water is used for washing the fillets after skinning, trimming and parasite

checking steps. In some cases, the fillets are then treated with additives in tumblers. Examples of additives which are often used to treat *Pangasius* fillets include sodium tri-poly phosphate or cryoprotectant (Sorensen, 2005).

The fillets are then graded manually according to weight into four groups: 60-120 g, 120-170 g, 170-220 g and ≥ 220 g per fillet. Before freezing, the fillets are placed into plastic bags and cooled with flake ice. During the freezing process, the individual fillets are manually placed into an Individual Quick Freezer (IQF) and frozen until a core temperature of -18°C. The frozen products are then packed into cardboard boxes, labeled and stored at -18°C. The entire production process from raw *Pangasius* fish to frozen fillets takes around 4.5 hours. With regard to yields, it has been estimated that 2.6 kg of fresh *Pangasius* fish are required to produce 1 kg of individually quick-frozen *Pangasius* fillets (Anh et al., 2010).

1.2.2.2 Quality properties of Pangasius

Vietnamese *Pangasius* products are currently exported to 145 countries in the world (VASEP, 2013). This has largely been a result of their reasonable cost and their 'white flesh', but also due to their good nutritional quality (Karl et al., 2010; Orban et al., 2008). The good nutritional quality of *Pangasius* is reflected in its proximate composition, a good source of amino acids, low residue levels of heavy metals and polychlorinated biphenyls, etc. The proximate compositions of Vietnamese farmed *Pangasius* product sold in Italy, Germany and Poland were similar with 12.9-15.7% protein, 1.3-3.2% fat, 0.8-1.3% ash and 82.1-84.7% water (Karl et al., 2010; Orban et al., 2008; Szlinder-Richert et al., 2011; Van Leeuwen et al., 2009). Furthermore, the composition of the lipids has been studied intensively, the results of which indicate that they consist of high amounts of saturated fatty acids (41.1-47.8% of total fatty acids), low amounts of polyunsaturated fatty acids (12.5-24% of total fatty acids) with mainly linoleic acid (15-17% of total polyunsaturated fatty acids) and low cholesterol levels (21-39 mg/100 g) (Karl et al., 2010; Orban et al., 2008; Usydus et al., 2011). Regarding polyunsaturated fatty acids, eicosapentaenoic acid (EPA, C20:5, *n*-3) and docosahexaenoic acid (DHA, C22:6, *n*-3), which have been reported to have beneficial health effects, particularly in the prevention of cardiovascular diseases (Kris-Etherton et al., 2002), were found in *Pangasius* at levels of 3.7 and 21.1 mg/100 g of muscle, respectively (Usydus et al., 2011). However, the quantity of EPA+DHA in Vietnamese *Pangasius* (24.8 ± 5.7 mg/100 g) is much lower than that found in Baltic salmon (3807 ± 666 mg/100 g), farmed Polish trout

(1804 ± 279 mg/100 g) and Baltic herring (941 ± 307 mg/100 g) marketed in Poland (Usydus et al., 2011). Fat-soluble vitamins such as vitamin A, D_3 and E have been reported at levels of 1.61 ± 0.54 µg/100 g, 0.31 ± 0.01 µg/100 g and 0.2 ± 0.05 mg/100 g, respectively (Szlinder-Richert et al., 2011).

Sodium occurs at high levels between 222-594 mg/100 g (Karl et al., 2010; Orban et al., 2008). These high levels were demonstrated (by means of differential scanning calorimetry) to be mostly likely a result of the use of sodium polyphosphate during processing for water retention purpose. The maximum amount of polyphosphates allowed in the EU to be added to deep frozen fish fillets is 5 g/kg (calculated as P_2O_5); therefore, it has been recommended that *Pangasius* fish destined for export to the EU in which polyphosphates are used should be labeled accordingly (Karl et al., 2010). Calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) has been found to range between 80-90, 1700-3400, 4000-6000 and 120-170 µg/g, respectively (Orban et al., 2008; Ruiz-de-Cenzano et al., 2013). Regarding toxic metals, the level of mercury (Hg) in *Pangasius* was determined to vary largely between various studies i.e. 0.4 (Ferrantelli et al., 2012), 0.3 (Orban et al., 2008), 0.005 (Usydus et al., 2011) and 0.002 µg/g (Ruiz-de-Cenzano et al., 2013). These levels are however below the tolerable level of 0.5 µg/g stipulated by European Commission (EU, 2006). Furthermore, the levels of cadmium (Cd) and lead (Pb) in *Pangasius* samples has been determined to be lower than the maximum limits (Cd = 0.05 µg/g and Pb = 0.3 µg/g) stipulated by the European Commission (Ruiz-de-Cenzano et al., 2013; Szlinder-Richert et al., 2011).

In addition to the quantity of protein in *Pangasius*, the quality of the protein has also been evaluated. The digestible proteins of *Pangasius* from Vietnam have been determined to be rich in essential amino acids (Szlinder-Richert et al., 2011). Total essential amino acid and non-essential amino acid levels were determined to be *ca*. 5.91 and 6.53 g/100 g fish, respectively. The essential amino acid content in 100 g fish was comparable with the levels of these amino acids recommended for an adult human weighting 70 kg (5.91 compared to 5.59 g/100 g) (**Table 1.2**). Moreover, as can be seen in **Table 1.3** the essential amino acid content of *Pangasius* muscle proteins (total = 45.6 g/100 g protein) is greater than that of the reference protein suggested by the FAO/WHO (total = 32 g/100 g protein) (FAO/WHO, 1991). These values are similar to those of other appreciated fish i.e. Baltic cod (44.2 g/100 g protein) and trout (46.1 g/100 g protein) (Szlinder-Richert et al., 2011). The protein of *Pangasius* is therefore considered as a good source of essential amino acids.

Table 1.2 (Non) essential amino acid compositions in *Pangasius* fish (Szlinder-Richert et al., 2011)

Essential Amino acids (AA)	Recommended daily intake		g/100 g product	Non-essential amino acids	g/100 g product
	Body weight (mg/ kg)	Body weight (g/ 70 kg)			
Phenylalanine + Tyrosine	12.1	0.85	1.00	Alanine	0.72
Isoleucine	15.7	1.10	0.65	Arginine	0.84
Leucine	9.5	0.67	1.07	Glycine	0.65
Lysine	9.4	0.66	1.37	Histidine	0.30
Methionine + Cysteine	12.1	0.85	0.54	Aspartic acid	1.33
Threonine	6.5	0.46	0.56	Glutamic acid.	1.87
Tryptophan	2.9	0.20	0.12	Proline	0.36
Valine	11.4	0.80	0.60	Serine	0.46
Total essential AA	**79.6**	**5.59**	**5.91**	**Total non-essential AA**	**6.53**

Table 1.3 Comparison of essential amino acid levels in *Pangasius* muscle protein to the reference protein composition recommended by the FAO/WHO (Szlinder-Richert et al., 2011)

Essential Amino acids	Standard FAO/WHO (1991)	g/100 g protein
Phenylalanine + Tyrosine.	6.3	7.7
Isoleucine	2.8	5.0
Leucine	6.6	8.3
Lysine	5.8	10.6
Methionine + Cysteine	2.5	4.2
Threonine	3.4	4.3
Tryptophan	1.1	0.9
Valine	3.5	4.6
Total Essential AA	**32.0**	**45.6**

It has to be taken into consideration that *Pangasius* fish can contain undesirable substances including antibiotics such as nitrofuran and/or its metabolites and chloramphenicol. These drugs were banned to protect consumers from antimicrobial resistance and other adverse

effects. Therefore, residues of these drugs are not permitted to be present in *Pangasius* products imported by the European Union and the U.S (FDA, 2009; Sarter et al., 2007). Testing for the presence of residues of these compounds is recommended 3-4 weeks before harvesting of the fish and during processing under the instructions of the National Fisheries Quality Assurance and the Veterinary Directorate in Vietnam (Cuyvers and Van Binh, 2008). *Pangasius* fillets marketed in Poland were monitored by Szlinder-Richert et al. (2011). The presence of nitrofuran and its metabolites [AOZ (3-amino-2-oxazolidin), AMOZ (3-amino-5-morpholinomethyl-2-oxazoline, AHD (1-aminohydantoin), SEM (semicarbazide)] and chloramphenicol antibiotics occurred in the *Pangasius* fillets only at trace value (<1 and <0.3 µg/kg, respectively) and are therefore lower the Minimum Required Performance Limits (MRPL) for import products based on the recommendation of the European Commission in 2004 (EU, 2004a). The European Commission operates a Rapid Alert System for Food and Feed program which (amongst other activities) pro-actively informs member states about problems or risk concerning food and fish which do not meet the sanitary requirements. Based on the database of Rapid Alert System for Food and Feed (RASFF., 2013), there were 7 cases of border rejection by European countries from 2002 to 2012 due to the detection of nitrofuran (and its metabolites) in imported *Pangasius* at levels which ranged from <0.4 to 2.3 µg/kg. In addition, 7 cases were also reported during the same period of chloramphenicol in *Pangasius* products exported from Vietnam. The levels of chloramphenicol were reported from 0.1 to 1.0 µg/kg.

In contrast, toxic halogenated environmental contaminants (i.e. polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins and dibenzo-p-furans, organochlorinepesticides, polybrominated diphenyl ethers, hexabromocyclododecane diastereomers, and perfluorinated compounds), which can cause cancer in animals including humans, have been determined to occur in Vietnamese *Pangasius* products at levels (<1ng/g), which are far below European and Dutch legislative limits (Van Leeuwen et al., 2009).

1.3 Microbiota of *Pangasius* and freshwater fish

Due to the increasing popularity of fish and fishery products, attention has been focused on the quality and safety. Regarding the shelf life of fishery products, rapid deterioration of fish is caused mainly by spoilage microorganisms leading to reduced shelf life and economic loss. On the other hand, outbreaks of foodborne illness can occur due to consuming products contaminated with pathogenic bacteria. Therefore, the types of spoilage microorganisms and

pathogenic microorganisms contaminating freshwater fish in general and *Pangasius* in particular and their origin will be discussed here.

1.3.1 Microbial ecology of freshwater fish

The microbiota of freshwater fish species (i.e. *Pangasius*, tilapia, rainbow trout, silver perch, striped bass, etc.) varies depending on the condition of the water (polluted *vs.* unpolluted water), temperature, antibiotics used and feeding methods (wild *vs.* active feeding) (Le Nguyen et al., 2008; ICMSF, 2005). The initial counts of the microbiota on freshly harvested, properly handled pond reared fish products are similar to those on wild fish. It is reported that Gram-negative bacteria are dominant on farmed and tropical fish. The nature of the microbiota on skin, gut and living freshwater fish is summarized in **Table 1.4.**

Table 1.4 The microbial ecology of different parts of freshwater fish

Freshwater fish organs	Bacterial counts Log CFU/cm^2 or CFU/g	Bacterial Genera	References
Skin	2-4	**Gram-negative bacteria:** *Acinetobacter, Aeromonas, Alcaligenes, Enterobacter, Flavobacterium, Flexibacter, Pseudomonas, Psychrobacter, Citrobacter,* and *Moraxella* spp. **Gram-positive bacteria:** *Micrococcus, Staphylococcus,* and *Streptococcus* spp.	(Apun et al., 1999; Austin, 2002; Ghaly et al., 2010; ICMSF, 2005).
Gut	2-9	**Gram-negative bacteria:** *Acinetobacter, Aeromonas, Citrobacter, Enterobacter, Escherichia, Klebsiella, Vibrio, Pseudomonas* spp., *Serratia, Flavobacterium, Moraxella* spp. **Gram-positive bacteria:** *Bacillus, Listeria, Staphylococcus, Streptococcus,Micrococcus, Corynebacterium,Carnobacteria, Lactobacillus, Enterococcus,* and *Vagococcus* spp.	(Apun et al., 1999, Austin, 2002; ICMSF, 2005).
Living fish	2-6	**Gram-negative bacteria:** *Acinetobacter, Aeromonas, Pseudomonas, Shewanella, Flavobacterium, Psychrobacter,* and *Moraxella* spp. **Gram-positive bacteria:** *Bacillus, Micrococcus, Clostridium, Lactobacillus* and *Corynebacterium* spp.	(ICMSF, 2005).

Sarter et al. (2007) identified isolates collected from the intestines and gills of 92 Vietnamese *Pangasius* fish (from 3 different farms) by means of API System strips. The identified isolates included *Enterobacteriaceae* (49.1%), *pseudomonads* (35.2%) and *Vibrionaceae* (15.7%). Particularly, the incidence of *Enterobacteriaceae* has been reported to be higher on pond-reared and tropical fish than on marine fish (ICMSF, 2005).

1.3.2 Fish spoilage and microbiota related to spoilage

Generally, the quality and hygiene of any aquatic product depends on the characteristics of the production chain including (i) the quality of water in which the fish are reared, (ii) the composition and safety of the feed they are given, (iii) handling of the fish during processing, (iv) transportation and storage conditions, and (v) conditions during retail and storage up to the point the fish is on the consumer's table (Orban et al., 2008). Regarding the quality of fishery products, fresh fish spoilage can be very rapid after the fish death due to enzymatic, chemical and bacteriological activities. The mechanisms of fish spoilage and preservation techniques of freshwater fish and/or *Pangasius* will be discussed here.

1.3.2.1 Fish spoilage mechanisms

The enzymatic and chemical reactions are usually responsible for the initial loss of quality while microbial activity is responsible for subsequent spoilage (Ghaly et al., 2010; Mohan et al., 2008). Chemical spoilage is related to oxidation phenomena like rancidity caused by (non) enzymatic oxidation. On the other hand, the intensive enzyme activity in the intestinal tract (gut) of fish digests the wall of the gut soon after death. The enzymatic autolysis and proteolysis (i.e. cathepsins, peptidase, trypsin, calpain, etc.) can cause bursted belly and softens the tissue as a result of degradation of proteins. Bursting of the belly permits microorganisms to enter the flesh surrounding the belly (ICMSF, 2005). The reason of bacteriologically spoilage is that at death and during evisceration and filleting, endogenous micro-organisms from the raw material may contaminate the sterile fish flesh. In addition to the microbial contaminants originating from the fish itself, the microorganisms from the processing environment (working tables, equipment etc.) as well as manual manipulation are a source of contamination (Chen et al., 2010; Norton et al., 2001). More specifically, as presented in **Figure 1.9**, the spoilage of fish includes four phases: phase 1 and 2 due to autolytic deterioration caused by enzymatic activity, phase 3 and 4 due to bacteriological activities. The quality changes of fish stored on ice are induced by enzymatic activity from

phase 1 with slight loss of natural odor and flavors (about 1 to 2 days) to phase 2 with significant loss of natural flavor and odor of fish but texture is still pleasant (till 6 days). Subsequently, bacterial activity is responsible for spoilage processes until the end of the shelf life (= the moment of phase 3) where the textural changes are significant, and strong off flavors and unpleasant smell occurs) and further spoilage (where the fish becomes completely spoiled and become inedible = phase 4) (Shawyer and Pizzali, 2003). The growth and metabolism of contaminating bacteria result in the formation of amines, sulfides, alcohols, aldehydes, ketones and organic acids with unpleasant and unacceptable off-favors (Dalgaard et al., 2006; Ghaly et al., 2010; Gram et al., 2002).

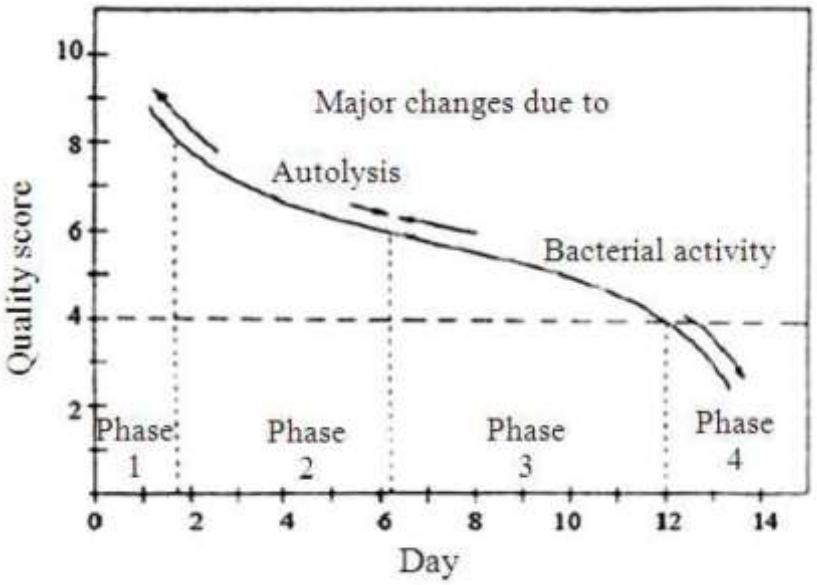


Figure 1.9 Four phases of fish spoilage (Shawyer and Pizzali, 2003)

1.3.2.2 Some preservation techniques

Preservation methods for extending the shelf life of fresh fish range from mild chemical treatments (i.e. the application of sodium acetate, tannic acid etc.), vacuum and modified atmosphere packaging (MAP), bio-preservation (the use of a natural or controlled microflora and/or its antimicrobial metabolites i.e. lactic acid bacteria producing *in-situ* antimicrobial compounds such as diacetyl, bacteriocins etc.), low temperature (i.e. at 0, 2, 4 and 7°C) to freezing (Ghaly et al., 2010; Gram and Dalgaard, 2002; Gram and Huss, 1996; Maqsood and Benjakul, 2010; Yesudhason et al., 2010). The frozen storage can prolong the shelf life of fishery products more than one year due to retarding the activity of spoilage bacteria under non-fluctuating storage temperature lower than – 30°C (Jessen et al., 2013). Due to a few

studies on *Pangasius* fish up to date, only one study of thawed *Pangasius* fillets stored under MAP conditions. On the other hand, MAP is a well-known, commonly applied storage method to extend the shelf life of perishable fishery products. MAP is defined as the enclosure of food products in packaging materials in which the gas composition ($\%O_2$, $\%CO_2$, $\%N_2$) inside the package has been altered (Sivertsvik et al., 2002). In most cases, MAP employs elevated CO_2 and/or reduced O_2 concentrations to selectively inhibit chemical, enzymatic and microbial spoilage. Whilst low O_2 levels suppress the growth of aerobic spoilage bacteria, they can enhance the growth of anaerobic and microaerophilic bacteria such as lactic acid bacteria. Therefore, the composition of the atmosphere used in a modified atmosphere depends on several factors including the characteristics of products, types of spoilage microorganisms, the desired shelf life, etc. MAP gives positive effects to extend the quality; maintain the hygienic and sensory properties; and transport to far distance places (Torrieri et al., 2011; Yesudhason et al., 2009). The use of MAP to extend the shelf life of marine fish has been studied widely whilst its application to the preservation of freshwater fish is less documented. In addition to MAP *Pangasius* products, recent studies investigating the application of MAP to freshwater fish were summerised in **Table 1.5.** In general, the shelf life of stored fish is normally determined by means of sensory evaluation (appearance, color, odor, slime, etc.) and the microbiological quality. In particular for MAP fish, the point of sensorial rejection may already have been reached at a total aerobic count of 7 to 8 log CFU/g (Dalgaard et al., 2002).

Table 1.5 Overview of the shelf life of freshwater fish products stored under different modified atmosphere package (MAP) conditions and different temperatures

Products[1]	**Country**	**Storage temp.**	**Atmosphere % $CO_2/O_2/N_2$**	**Microorganisms enumerated**	**Shelf life (days)**[2]	**Dominant spoilage bacteria identified**	**Methods of identification for spoilage bacteria**[3]	**References**
Farmed *Pangasius* fillets	Vietnam	4°C	Air	Total counts H_2S producing bacteria *pseudomonads*, Lactic acid bacteria *B. thermosphacta*	7	*Pseudomonas synxantha* *Pseudomonas trivialis* *Serratia proteamaculans* *Serratia grimesii*	16S rRNA sequence	(Noseda et al., 2012)
Farmed *Pangasius*	Vietnam	4°C	50/50/0	Total counts H_2S producing bacteria *pseudomonads*, Lactic acid bacteria *B. thermosphacta*	14	*B. thermosphacta* *Carnobacterium maltaromaticum* *C. divergens*	16S rRNA sequence	(Noseda et al., 2012)
Farmed *Pangasius*	Vietnam	4°C	Vacuum	Total counts H_2S producing bacteria *pseudomonads*, Lactic acid bacteria *B. thermosphacta*	10	*Serratia quinivorans* *Serratia fonticola* *S. proteamaculans* *C. maltaromaticum* *Pseudomonas mephitica*	16S rRNA sequence	(Noseda et al., 2012)
Farmed *Pangasius*	Vietnam	4°C	50/0/50	Total counts *pseudomonads*, Lactic acid bacteria *B. thermosphacta*	12	*C. maltaromaticum* *B. thermosphacta* *Serratia glossinae*	16S rRNA sequence	(Noseda et al., 2012)
Farmed eel	Greece	0°C	40/30/30	Total counts *pseudomonads*, *Enterobacteriaceae* Lactic acid bacteria H_2S producing bacteria Yeasts	34			(Arkoudelos et al., 2007)
Farmed eel	Greece	0°C	Air	Total counts *pseudomonads*, *Enterobacteriaceae* Lactic acid bacteria H_2S producing bacteria Yeasts	18			(Arkoudelos et al., 2007)

Table 1.5*(continued)*

Products[1]	**Country**	**Storage temp.**	**Atmosphere % $CO_2/O_2/N_2$**	**Microorganisms enumerated**	**Shelf life (days)**[2]	**Dominant spoilage bacteri identified**	**Methods of identification for spoilage bacteria**[3]	**References**
Farmed eel	Greece	0°C	Vacuum	Total counts *pseudomonads, Enterobacteriaceae* Lactic acid bacteria H_2S producing bacteria Yeasts	28			(Arkoudelos et al., 2007)
Farmed Tilapia	China	4°C	Air	Total counts	6	*pseudomonads* *Aeromonas* *Staphylococcus*	Biochemical tests combined with taxonomic scheme	(Cao et al., 2012)
Farmed Tilapia	Thailand	4°C	Air	Total counts	6			(Masniyom et al., 2013)
Farmed Tilapia	Thailand	4°C	35/5/60	Total counts	15			(Masniyom et al., 2013)
Farmed Tilapia	Thailand	4°C	Vacuum	Total counts	12			(Masniyom et al., 2013)
Pearl spot	India	0-2°C	Air	Total counts	8			(Manju et al., 2007)
Pearl spot	India	0-2°C	Vacuum	Total counts	10			(Manju et al., 2007)
Pearl spot	India	0°C	Air	Total counts *B. thermosphacta* Lactic acid bacteria H_2S producing bacteria Yeasts and moulds	12-14	*Pseudomonas spp.* *Aeromonas spp.* *Shewanella* spp.	Biochemical tests combined with taxonomic scheme	(Lalitha et al., 2005)

Table 1.5 *(continued)*

Products[1]	**Country**	**Storage temp.**	**Atmosphere % $CO_2/O_2/N_2$**	**Microorganisms enumerated**	**Shelf life (days)**[2]	**Dominant spoilage bacteria identified**	**Methods of identification for spoilage bacteria**[3]	**References**
Pearl spot	India	0°C	60/40/0	Total counts *B. thermosphacta* Lactic acid bacteria H_2S producing bacteria Yeasts and moulds	21	*B. thermosphacta* *Shewanella* spp	Biochemical tests combined with taxonomic scheme	(Lalitha et al., 2005)
Gutted Pabda catfish	India	4 ± 2°C	Vacuum	Total Viable Count, Enterobacteriaceae, *Escherichia coli*, *Salmonella* Spp, Total vibrios, *L. monocytogenes*, *S. aureus*, Faecal *Streptococcus* anaerobic sulphite reducers	18-20			(Binsi et al., 2013)
Gutted Pabda catfish	India	4 ± 2°C	Air	Total Viable Count, *Enterobacteriaceae* , *Escherichia coli*, *Salmonella* Spp, Total vibrios, *L. monocytogenes*, *S. aureus*, Faecal *Streptococcus* anaerobic sulphite reducers	14-16			(Binsi et al., 2013)

[1] All products were in fillet form with the exception of the whole eel and pearlspot, [2] Total microbial counts of 7 log CFU/g are used to determine shelf life, [3] Method for identification of dominant spoilage bacteria.

1.3.2.3 Microbiota related to spoilage of fresh water fish

The growth of dominant bacteria depends on the fish species, storage temperature and modified atmosphere conditions. It is reported that Gram–negative bacteria are considered to be dominating spoilage bacteria of tropical fish (such as *Pseudomonas* and *Acinetobacter* spp.) (ICMSF, 2005) and of aerobic-chilled fish (such as *Pseudomonas* and *Shewanella* spp.) (Gram and Dalgaard, 2002). To date, only one study has been performed on the microbiological spoilage of vacuum and MAP packed Vietnamese *Pangasius*. The dominant microbiota on thawed *Pangasius* fillets stored under different conditions were determined to be *Pseudomonas* and *Serratia* spp. (in air), *Brochothrix thermosphacta* and *Carnobacterium* spp. (in 50% CO_2 + 50% O_2), *B. thermosphacta, Serratia* and *Carnobacterium* spp. (in 50% CO_2, 50% N_2) and *Serratia* and *Carnobacterium* spp. (in vacuum) (Noseda et al., 2012). These bacteria were determined to produce various volatile metabolites such as ethanol, 2,3-butanediol, diacetyl, acetoin, ethyl acetate, acetic acid and sulfur compounds like hydrogen sulfide, methyl mercaptan, carbon disulfide and dimethyl disulfide. These volatiles were considered as potential indicators of spoiled *Pangasius*. Some characteristics of dominant spoilage microorganisms isolated from freshwater Vietnamese *Pangasius* are briefly discussed below.

Pseudomonads are well known as a widespread environmental group of bacteria (Bagge-Ravn et al., 2003). They are dominant specific spoilage bacteria of chilled tropical fresh-water fish stored under aerobic conditions (Ghaly et al., 2010; Gram et al., 2002). *Pseudomonas* spp. produce a number of volatile aldehydes, ketones, esters and sulphides (Church, 1998). Therefore, fruity, rotten, sulphydryl odors and flavours are typical of the spoilage of iced fish by *Pseudomonas* spp. (Gram and Melchiorsen, 1996; Gram et al., 2002).

Unlike *Pseudomonas* spp., which are inhibited under anaerobic conditions (Devlieghere and Debevere, 2000), Gram-positive lactic acid bacteria (LAB) (e.g. *Carnobacterium*) are tolerant to CO_2. Although *Carnobacterium* are not common in freshwater fish products (Gonzalez et al., 2000; Ringø and Gatesoupe, 1998), they can be present as a result of (cross) contamination during processing (Noseda et al., 2012; Ringø and Gatesoupe, 1998; Vijayabaskar and Somasundaram, 2008). They are often found as the dominating spoilage organisms on vacuum and MAP freshwater fish (Arkoudelos et al., 2007; Noseda et al., 2012).

Serratia spp. are considered as moderate spoilers which can grow well under both anaerobic and aerobic conditions. *Serratia* spp. have been isolated from thawed *Pangasius* (Noseda et al., 2012), smoked rainbow trout (Lyhs et al., 1998), salmon at onset of spoilage (Macé et al., 2013), cooked and peeled tropical shrimps (Jaffrès et al., 2011) stored under MAP. *Serratia* spp. have been determined to produce a mixture of spoilage odors including sour and cheesy compounds and to produce amines (Macé et al., 2012).

B. thermosphacta, a Gram-positive bacterium more common in meat products, also plays an important role in the spoilage of MAP shrimp (Jaffrès et al., 2009; Laursen et al., 2006; Mejlholm et al., 2005), and fish (Joffraud et al., 2001; Macé et al., 2012; Macé et al., 2013). *B. thermosphacta* has been reported to contribute to a lesser extent than *Pseudomonas* spp. to the spoilage of sea bass stored in ice (Papadopoulos et al., 2003). In general, although the initial counts of *B. thermosphacta* are low, they become dominant towards the end of storage of MAP fish. In addition, it has been reported that *B. thermosphacta* can cause spoilage at low counts (<7 log CFU/g). Therefore, their presence (even at low numbers) has to be taken into account (Drosinos et al., 1996; Noseda et al., 2012).

1.3.3 Microbial pathogens associated with fish and fishery products

The microbial hazards associated with aquatic products include bacteria, viruses and parasites. Among viruses, it is well known that norovirus is the most common cause of acute gastroenteritis as well as a great threat to the safety of edible (shell)-fish worldwide. It is normally transmitted by person-to-person contact and contaminated sewage. In addition, their presence in fishery products is a consequence of poor hygiene because they are transmitted either by contaminated water or by food handlers (Li et al., 2009; Weinstein et al., 2008). The detection of viruses in fish is relatively difficult due to their occurrence at low numbers; therefore, there is a genuine need for the development of improved techniques to detect viruses which would enable a better understanding of the role of viruses in foodborne illnesses associated with the consumption of fish and fishery products (McCoy et al., 2011; Tuan et al., 2010). Parasites can be transmitted through contaminated water and fish and cause a disease when raw or undercooked fish is consumed (Chai et al., 2005; Slifko et al., 2000). Although a large number of parasites infect fish, a few cause illnesses in humans (Lima dos Santos and Howgate, 2011; McCoy et al., 2011). Parasites such as trematode (*Opisthorchis felineus, O. viverrini*, and *Clonorchis sinensis*), larvae (*Anisakis simplex*),

tapeworms (*Diphyllobothriumi)* and round worms (*Anisakis* and *Pseudoterranova*) are typically found on fishery products from warm water areas (ICMSF, 2005). Bacterial pathogens are naturally present in aquatic environments, animals, humans etc. Bacterial pathogens originating from the environment include *Listeria monocytogenes*, *Clostridium botulinum, Clostridium perfringens, Vibrio parahamolyticus* (positive for thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) genes), *Vibrio vulnificus* (e.g. biotype 1), *Vibrio cholerae* (e.g. pathogenic serotypes O1, O139) and *Bacillus cereus* (only toxigenic strains, e.g. hemolysin – HBL positive strains), whilst those originating from people and animals include *Salmonella, Shigella* spp., pathogenic *Escherichia coli* (i.e. shiga toxin producing *E. coli* (STEC) O157:H7) and *Staphylococcus aureus* (Onjong et al., 2014; Sivertsvik et al., 2002). In addition to these primary bacterial pathogens, some opportunistic pathogens (mainly causing infections particularly in children, elderly and immunocompromised persons) include *Plesiomonas shigelloides, Aeromonas hydrophila, Acinetobacter baumannii, Pseudomonas aeruginos, Stenotrophomonas maltophilia, Vibrio vulnificus* (biotype 2) etc. Contamination by these pathogenic bacteria can occur at any point during the entire production chain of preharvest, capture, processing, distribution and storage (Venugopal, 2002). The prevalence of pathogens on freshwater fish has been well documented. Papadopoulou et al. (2007) determined the incidence of pathogens on 100 freshwater fish products in Greece. *A. hydrophila* (incidence = 38%), *E. coli* (14%), *S. aureus* (6%), *Plesiomonas shigelloides* (2%), *C. perfringens* (1%), and others were isolated from the fish products. Yucel and Balci (2010) reported a high incidence of *L. monocytogenes* (44.5%) on 30 samples of freshwater fish marketed in Turkey. A high prevalence of *V. cholerae* was found in catfish samples (33 positive out of 120 samples, incidence = 27.5%) during summer and autumn while none of these fillets sampled during winter and spring was positive for *V. cholerae* (Fernandes et al., 1997). The highest incidences of pathogens were found on the skin samples. Whole and filleted catfish has been associated with pathogens such as *A. hydrophila, E. coli, Listeria* spp., *P. shigelloides, S. aureus* and *V. parahaemolyticus,* etc. (Ramos and Lyon, 2000).

In **Table 1.6** summarizes all documented cases of pathogenic bacteria on frozen *Pangasius* products originating from Vietnam and imported in the EU during the period between January 2005 and December 2013. The highest number of contaminated samples occurred in 2009, where 22 notifications of *L. monocytogenes* and *Salmonella* spp. were reported. The presence of *V. cholerae* on *Pangasius* products was also reported in 2008. A high

contamination level of *E. coli* (1×10^2 - 4.9×10^2 CFU/g) was also reported on imported *Pangasius* products in 2007 and 2012 (RASFF., 2013).

Table 1.6 Notifications of frozen *Pangasius hypoththalmus* imported into EU originating from Vietnam contaminated with pathogenic bacteria from 1-2005 to 12-2013 (RASFF., 2013)

Year	Notifying country	Pathogens	Number of notifications	Action taken
2005	Italy	*L. monocytogenes*	19	Destruction/ Border rejection
	France	*L. monocytogenes*	1	Destruction
2007	Spain	*E. coli*[*] (3×10^2 CFU/g)	1	No distribution
2008	Bulgaria	*V. cholerae* NON-O1/NON-O139	2	No distribution
	Norway	*V. cholerae* NON-O1/NON-O139	1	No distribution
2009	Greece	*L. monocytogenes*	10	Border rejection
	Italy	*Salmonella Nottingham*	1	Border rejection
	Italy	*L. monocytogenes*	1	Border rejection
	Sweden	*Salmonella* spp.	1	Border rejection
	Bulgaria	*L. monocytogenes*	8	Border rejection
	Spain	*Salmonella* spp.	1	Border rejection
2010	Italy	*L. monocytogenes*	1	Border rejection
	Italy	*Salmonella enteritidis*	1	Border rejection
	Romania	*L. monocytogenes*	1	Border rejection
	Poland	*L. monocytogenes*	10	Border rejection
	Spain	*L. monocytogenes*	2	Border rejection
	Germany	*Salmonella* spp.	1	Withdrawn from markets
2011	Latvia	*L. monocytogenes*	1	No distribution
2012	Germany	*E. coli*[**] (1×10^2-4.9×10^2 CFU/g)	1	Border rejection
2013	Sweden	*Salmonella* spp.	1	Informing recipients

[**]Samples were altered organoleptic characteristics combined with high counts of *E. coli*

[*, **] no specific pathogenic strains of *E. coli* was noticed.

In general, *L. monocytogenes, V. cholerae, and Salmonella* spp. have been the most frequently isolated pathogens on Vietnamese *Pangasius* products imported during this 8-year period. Therefore, the following discussions will focus on these pathogens.

The genus *Vibrio* is comprised of Gram-negative, oxidase-positive, facultative anaerobic rods. The main species of importance with respect to foods are *V. cholerae*, *V. parahaemolyticus*

and *V. vulnificus*. In tropical and temperate regions, these species occur naturally in marine and coastal environments. In particular, *V. cholerae* can be recovered from freshwater environments (Dalsgaard, 1998). The contamination of food production environments such as ponds by faeces can indirectly introduce *V. cholerae* into food. No evidence has been observed which confirms that *Vibrio* spp. are present in the intestinal tracts of farmed channel catfish (Macmillan and Santucci, 1990). *V. cholerae* serogroup O1 and O139 producing cholera toxin or non-O1 strains (causing weak gastroenteritis) have an estimated minimal infectious dose from 3 log to 8-9 log CFU/g (Feldhusen, 2000). The *Vibrio* species mentioned above constitute a considerable risk for persons consuming raw molluscs and shellfish (Ahmed, 1992; Su and Liu, 2007).

L. monocytogenes is a Gram-positive rod shaped facultative anaerobic bacterium. *L. monocytogenes* is widely distributed in the environment (soil, water) and the occurrence of *L. monocytogenes* in raw and processed foods has been intensively studied. In a relatively recent study, *L. monocytogenes* is reported with high prevalence on catfish fillets i.e. to be 37.4% of 240 samples collected from three processing plants at U.S. (Chou et al., 2006) and 23.5% of 272 samples collected at local (central Virginia, U.S.) and through Internet (U.S.) retail markets (Pao et al., 2008). The *L. monocytogenes* on the processed catfish fillets has however reported to originate from the processing environment as no *L. monocytogenes* has been isolated from the skins and intestines of the catfish (Chen et al., 2010). Moreover, as *L. monocytogenes* is able to grow at refrigeration temperatures, its presence, even in low numbers (minimal infectious dose of 2 log CFU/g), may pose a risk to human health when fish products have been stored at refrigeration temperatures for long periods of time.

Salmonella spp. is a group of Gram-negative, rod shaped, facultative anaerobic bacteria, which are motile by means of flagella. *Salmonella* spp. are not indigenous to aquatic environments, being normally found in the intestinal tracts of animals and humans (Macmillan and Santucci, 1990). As an example, although *Salmonella* spp. were not isolated from catfish ponds, they were found in the guts and internal organs of freshwater fish (Gaertner et al., 2008; Macmillan and Santucci, 1990; Nesse et al., 2005). Fishery products can be contaminated by *Salmonella* when they are captured in polluted water, undergo inadequate (unhygienic) handling, and are distributed and retailed under poor conditions (Amagliani et al., 2012; McCoy et al., 2011; Panisello et al., 2000). It is reported that the incidence of *Salmonella* in 60 samples of channel catfish and Vietnamese basa (*Pangasius*

bocourti) fillets was 33% and 50%, respectively (Pal and Marshall, 2009). *Salmonella* spp. can cause gastrointestinal disease with a minimum infectiuos dose of 2 log CFU/g; however, human salmonellosis associated with the consumption of farmed freshfish is rare and there is a minimal risk to public health of fishery products that are cooked prior to consumption (Feldhusen, 2000).

1.4 Microbial quality management systems during processing

The typical characteristics of fish products such as high risks with regards to microbial safety, short shelf life, seasonal heterogeneity of raw materials and complex supply chains (domestic or international trade) result in high demands on systems used to control and/or assure the safety and quality of these products (Ababouch, 2006; Da Cruz et al., 2006; Luning and Marcelis, 2006). A good performance of a food safety management system (FSMS) can improve the microbiological quality and safety of food products (Kokkinakis and Fragkiadakis, 2007). Therefore, each fishery harvester and processor is required to use FSMS based on the Hazard Analysis Critical Control Point (HACCP) principles as HACCP is internationally accepted as a preventive food safety assurance system (CAC, 2003).

At the start of the 80s, many countries moved away from systems relying on the sampling of end products towards the application of HACCP-based food safety and quality systems. HACCP is the most effective system to control hazards during manufacturing because it relies on continuous monitoring and control of critical control points (CCPs) along the production chain (King, 2013). The use of hygienically designed equipment and pre-requisite programs (PRPs) such as Good Manufacturing Practices (GMP), Good Hygienic Practices (GHP) as well as the legislation are major components supporting any HACCP plan (Arvanitoyannis and Varzakas, 2009; Jacxsens et al., 2009a). A company specific FSMS is based on the translation of GHP, HACCP principles and available-relevant quality assurance guidelines and/or standards including legislation, guidelines on good practices of British Retail Consortium (BRC) and International Food Standard (IFS), etc. (CAC, 2003).

In Vietnam, up to date, there are 115 *Pangasius* processing companies (out of 621 seafood and freshwater fish companies in total). All companies processing *Pangasius* have implemented good manufacturing and hygiene practices (GMP and GHP), HACCP and/or additional standards such as BRC, IFS, and International Organization for Standardization (ISO) (i.e. ISO 9001, 22000, etc.) (NAFIQAD, 2015; VASEP, 2013).

The microbiological performance of a FSMS can be measured by means of a microbiological assessment scheme (MAS). A MAS is a vertical sampling plan applied throughout the production process (Jacxsens et al., 2009b). The principle behind MAS is that an effective FSMS results in lower contamination levels (compared to the acceptable level) and less variation in the contamination loads (Jacxsens et al., 2009b). As a microbiological safety assessment tool, the MAS is based on the collection and analysis for selected microbial indicator organisms in a restricted number of samples collected at critical sampling locations (Jacxsens et al., 2009b). In this way a MAS can be used to obtain the safety output of a FSMS. With this approach, analysis of various microbiological parameters on samples collected throughout the process from raw material to final products on different days can be used to establish the microbiological profile of a company-specific FSMS. Specifically, the critical sampling locations are locations where loss of control will result in unacceptable food safety problems due to contamination, growth and/or survival of microorganisms. The microbiological parameters are selected on the basis of the applicable national action limits, European legal criteria and knowledge of microbiological ecology of the products in question. Insight into the variability and distribution of microbial loads of each sampling point is obtained by collecting samples over three periods on each sampling day. FSMS self-assessment schemes developed by Jacxsens et al. (2011), Luning et al. (2008), Luning et al. (2009b) are comprised of context factors and a combined assessment of core control and assurance activities by means of a questionnaire (see **Table 1.7**). This assessment provides an independent evaluation of the performance of a FSMS in relation to the contextual situation wherein the FSMS operates. By two self-assessment tools, the current FSMS and the actual microbiological situation can be used to evaluate the food safety output. In addition, an overview of the results of MAS (= microbiological quality, hygiene, and safety level) can help quality managers to identify the strengths and weakness in the core control activities of an implemented FSMS (Luning et al., 2011b). MASs have been used to assess the microbiological performance of FSMS in a company producing ready-to-eat meals (Daelman et al., 2013), in the pork meat industry (Jacxsens et al., 2009b), in the lamb processing chain (Osés et al., 2012), in catering services (Lahou et al., 2012); and poultry slaughterhouses (Sampers et al., 2010).

Table 1.7 Context factors, core control and core assurance activities in a FSMS self-assessment scheme (Jacxsens et al., 2011; Luning et al., 2009a; Luning et al., 2008)

Context factors	**Core safety control activities**	**Core assurance activities**
Product characteristics	*Design preventive measures*	*Defining system requirements*
Risk of raw materials	Sophistication of hygienic design of equipment and facilities	Sophistication of translation of external requirements into FSMS
Risk of final product	Adequacy of cooling facilities	
Extent of safety contribution of packaging concept	Specificity of sanitation program	Extent of systematic use of feedback information to improve FSMS
Process characteristics	Extent of personal hygiene requirements	*Validation*
Extent of intervention steps	Adequacy of raw material control	Sophistication of validation of preventive measure
Degree of production process changes	Specificity of product specific preventive measures	
Rate of product/process design changes	*Design intervention processes*	Sophistication of validation of intervention systems
Organization characteristics	Adequacy of physical intervention equipment	
Presence of technological staff	Adequacy of packaging intervention equipment	Sophistication of validation of monitoring system
Variability in workforce composition	Specificity of maintenance and calibration for (intervention) equipment	
Sufficiency of operator competences	Specificity of intervention methods (chemical and biological)	*Verification*
Extent of management commitment	*Design monitoring system*	Extent of verification of people related performance
Degree of employee involvement	Appropriateness of CCP analysis	
Level of formalization	Appropriateness of standards and tolerances design	Extent of verification of equipment and methods related to performance
Sufficiency supporting information systems	Adequacy of analytical methods to assess pathogens	
Chain environment characteristics	Adequacy of measuring equipment to monitor critical process and product conditions	*Documentation and record-keeping*
Safety contribution in chain position	Specificity of calibration program for measuring and analytical equipment	Appropriateness of documentation system
Extent of power in supplier relationships	Specificity of sampling design (microbial assessment) and measuring plan	
Degree of authority in customer relationships	Extent of corrective actions	Appropriateness of record-keeping system
Severity of stakeholder requirements	*Operation control strategies*	
	Actual availability of procedures	
	Actual compliance to procedures	
	Actual hygienic performance of equipment and facilities	
	Actual cooling capacity	
	Actual process capability of physical intervention equipment	
	Actual process capability of packaging intervention equipment	
	Actual performance of measuring equipment	
	Actual performance of analytical equipment	

1.5 Intervention steps to control microbial quality during processing

During processing, an intervention step of washing is used to reduce or eliminate the microbial loads on the food products as much as possible. In some cases, wash water has been reused due to high cost of water (Luo, 2007). This practice results in the high risk of cross contamination from water to products. Therefore, water disinfection methods for washing practices are available such as chlorine, ozone, peracetic acid, hydrogen peroxide, etc. The evaluation of these water disinfection technologies to support processors the 'fit for purpose' especially for disinfection of vegetables is summarized by Van Haute et al. (2013c). As chlorine (a most common disinfectant) and peracetic acid (an alternative disinfectant) have been studied intensively on vegetables, poultries and fishery processing chains, the following discussions will therefore focus on chlorine and peracetic acid.

1.5.1 Chlorine

Chlorine-based products are the most widely used sanitization agents. They are used for both the treatment (decontamination) of food products and reducing the microbial load and/or to prevent the build-up of microorganisms on food contact surfaces, equipment and process water. Several types of chlorine compounds are used such as chlorine gas (Cl_2), hypochlorites (CaO_2Cl_2 or NaOCl) etc. The hypochlorite's are the most commonly used form of chlorine sanitizers in food manufacturing and food service. When chlorine is present in water, the chemistry of chlorine in solution can be basically described as follows:

$$NaOCl + H_2O \leftrightarrows HOCl + NaOH \qquad \text{Eq. 1}$$

$$Ca(OCl)_2 + 2H_2O \leftrightarrows Ca(OH)_2 + 2\,HOCl \qquad \text{Eq. 2}$$

$$Cl_2 + H_2O \leftrightarrows HOCl + H^+ + Cl^- \qquad \text{Eq. 3}$$

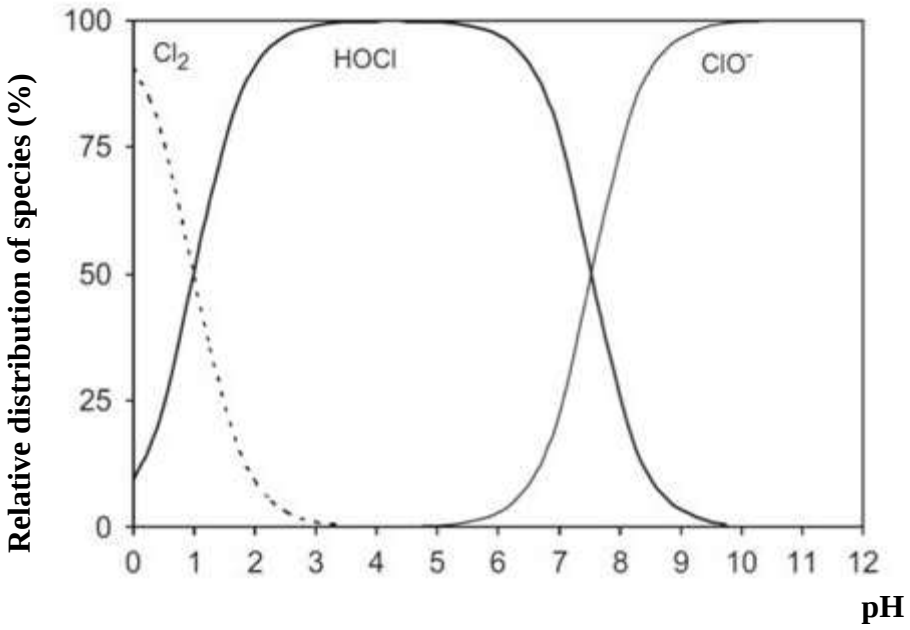


Figure 1.10 Distribution of chlorine species in aqueous solutions at 25°C with varying pH (Bruce et al., 2005)

The chlorine (or chlorinated) water consists of a mixture of three forms of chlorine: elemental chlorine (Cl_2), hypochlorous acid (HOCl) and hypochlorite ion (ClO^-) in amounts that vary with the pH of water. For example, a decrease in the pH (as from pH 4), results in an increase in the amount of Cl_2. Between pH 4 and 5, HOCl is the dominant species. Increase in pH (as from pH 5), results in an increase in the proportion of ClO^- (Cords et al., 2005) (**Figure 1.10**). Although all species (Cl_2, HOCl and ClO^-) show antimicrobial activity, hypochlorous acid is the most bactericidal in process water (Cords et al., 2005; Fonseca, 2006; Suslow, 2008). Several modes of action of antimicrobial activity by HOCl have been proposed including its effects on the cell membranes, the inhibition of sulfhydryl enzymes, the inhibition of enzymes involved in glucose metabolism, reaction with the DNA of living cells resulting in mutation by oxidation of purine and pyrimidine bases (Bruce et al., 2005; Dychdala, 2001). The antimicrobial effects of chlorine depend on the amount of free available chlorine present as HOCl. The dissociation of HOCl has been determined to be influenced by the pH, temperature and organic matter (Cords et al., 2005; Fonseca, 2006; Suslow, 2008).

Besides the terms free chlorine, total chlorine refers to the sum of the *free available* and *combined* chlorine (i.e. chlorine combined with ammonia or any oxidizable substrates) that are present in water and also ready for disinfection and oxidation of organic matter (Suslow,

2008). Although combined chlorine is more stable than free available chlorine, the former is a slower disinfection action than the latter. Generally, the disinfection process is controlled by monitoring the concentration of both free available and total chlorine. The difference between both values depends on the amount of organic and inorganic compounds in the water that can react with free chlorine to form combined chlorine. Higher concentration of organic and inorganic components result in a higher difference between the levels of free available and total chlorine (Virto et al., 2005).

The concentration of free chlorine used for decontamination of minimal processed vegetables ranges from 50 to 200 ppm (Beuchat, 1998) whilst that used for decontamination of *Pangasius* fish processing in Vietnam ranges from 2 to 10 ppm (Anh et al., 2010). The total residual chlorine concentrations recommended by Kanduri and Eckhardt (2008) for the sanitation of products and food contact surfaces in fish processing companies are shown in **Table 1.8**.

Table 1.8 Range of recommended concentrations of chlorine in water used for different purposes (Kanduri and Eckhardt, 2008)

Use	Recommended concentration (ppm)
Disinfection of products	2-10
Rinsing hands	50-100
Disinfection glazed surfaces	50-300
Disinfection smooth wood, metal or synthetic surfaces	300-500
Disinfection rough surfaces	1000-5000
Thawing/defrosting	5-10

The efficacy of chlorine has been studied for the decontamination of several products not only from the point of view of the microbiological quality but also with regards to its specific impact on the microbial safety. The decontamination efficacy of chlorine as NaOCl has been studied sporadically, particularly on vegetables. As can be seen in **Table 1.9** and **Table 1.10**, the decontamination efficacy on vegetables varied with regards to applied concentration and contact time, showing total counts and inoculated *E. coli*, respectively. However, very few studies have investigated the decontamination efficacy of chlorine on fish products. Bremer and Osborne (1998) reported that washing regimes with water containing chlorine at 200 mg/l at a turnover rate for the total wash solution of 2.25 cycles/h for 120 min could eliminate

over 2 log CFU/g of *L. monocytogenes* inoculated on the surface of gilled and gutted king salmon (*Oncorhynchus tshawytscha*) in industrial scale. Another study of Chaiyakosa et al. (2007) determined that chlorine at the concentration of 50 ppm and 30 min of contact time can reduce more than 1 log CFU/g of *V. parahaemolyticus* inoculated onto shrimp. As far as the author is aware, to date no chlorine decontamination studies have yet been performed to reduce the natural microbiota of fish.

Table 1.9 Reduction of total counts on vegetables by sodium hypochlorite

Microflora	**NaOCl Concentration** (ppm)	**Contact time** (min)	**Log reduction** Log (CFU/g)	**Product**	**References**
TPC*	200	2	1.7	Shredded carrot	(Klaiber et al., 2004)
TPC	200	1	1.6	Iceberg lettuce	(Sinigaglia et al., 1999)
TPC	100	1	1	Rocket leaves	(Martínez-Sánchez et al., 2006)
TPC	200	1	1	Shredded carrot	(Alegria et al., 2009)
TPC	50	/	1.1	Sugar snap	(Van Haute et al., 2013d)
TPC	200	/	1.4	Sugar snap	(Van Haute et al., 2013d)
TPC	200	5	2.63	Head lettuce	(Ha et al., 2013)
TPC	100	1	1.7	Iceberg lettuce	(López-Gálvez et al., 2010b)
TMC**	50	1	0.8	Iceberg lettuce heads	(Lopez-Galvez et al., 2013)
TMC	100	1	1.3	Iceberg lettuce	(López-Gálvez et al., 2010a)
TMC	200	1	1	Cut cilantro	(Allende et al., 2009)

/: mean value of 30 s, 60 s and 180 s; *TPC: total aerobic psychrotrophic counts; **TMC: total aerobic mesophilic counts.

Table 1.10 Reduction of *E. coli* inoculated on vegetables by sodium hypochlorite

Inoculated microflora	**NaOCl concentration** (ppm)	**Contact time** (min)	**Log reduction** Log (CFU/g)	**Product**	**References**
E. coli CECT 471, 516 and 533	40	1	2	Cut lettuce	(López-Gálvez et al., 2009)
E. coli O157:H7	200	1	1.3	Cut cilantro	(Allende et al., 2009)
E. coli O157:H7	200	2	2.5	Shredded carrots	(Ruiz-Cruz et al., 2007)
E. coli ATCC 10536	200	5	2.18	Cut lettuce	(Ha et al., 2013)
E. coli O157:H7	60	2	0.96	Cut-wash lettuce	(Palma-Salgado et al., 2014)
E. coli O157:H7	60	2	1.75	Wash-cut lettuce	(Palma-Salgado et al., 2014)
E. coli O157:H7	70	3	2.0	Mung bean sprouts	(Neo et al., 2013)

Though the chlorination process is reasonable with regards to cost and efficiency, the use of chlorine has some potential negative effects. Chlorine gas (Cl_2) itself may be harmful as it can irritate the skin and respiratory tracts of workers. In addition, the formation of hazardous disinfection by-products can be generated due to the use of excessive doses of chlorine and the reaction of chlorine with organic matter (Alegria et al., 2009). Under industrial conditions, the wash water is usually re-used to minimize water consumption (Luo, 2007). Consequently, the level of organic matter accumulates resulting in the deterioration of the water quality and microbial growth. A slight amount residual chlorine (i.e. 1 ppm) has therefore to be maintained in the washing system to avoid spread of contamination during product washing (Van Haute et al., 2013b). In this way, the quality of water as well as washed product can be improved. However, there is a high risk that disinfection by-products would be formed. In terms of disinfection by-products, two major classes are halogenated trihalomethanes (i.e. chloroform, bromodichloromethane, bromoform and dibromochloromethane) and haloacetic acids (monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid and dibromoacetic acid) which are commonly detected in water disinfected by chlorine (Gopal et al., 2007). In recent years, the potential formation of trihalomethanes in the process water and washed products has been studied by means of simulating industry-like operating conditions (**Table 1.11**). The concentration of trihalomethanes is limited to 80 µg/L in drinking water in U.S. (USEPA, 2009) and 100 µg/L in the EU (EU, 1998a). The maximum contamination levels of haloacetic acids, chlorite and bromate permitted in drinking water in U.S is 60, 1000 and 10 µg/kg, respectively (USEPA, 2009). European legislation emphasizes that water used in the food industry should have the same quality as water intended for human consumption. Therefore, the levels of trihalomethanes (THMs) in the process water should comply with those stipulated in the legislation for drinking water to avoid diminishing the wholesomeness of the finished products (EU, 1998a).

Table 1.11 Disinfection by-products evaluated on process water and washed products

Washing solution	Scale	Samples	Compounds				References
			THMs* **µg/g or L**	**Trichloromethane µg/g or L**	**Chloroform µg/L**	**Bromodichlo -romethane**	
NaOCl (100 ppm)+COD** (700ppm) for 30 min	Laboratory	Process water	217 ± 38	-	-	-	(López-Gálvez et al., 2010a)
NaOCl (100 ppm)+COD (700ppm) for 30 min		Washed lettuce	<5	-	-	-	
NaOCl (700 ppm)+COD (1800ppm) for 60 min		Process water	3618 ± 633	-	-	-	
NaOCl (700 ppm)+COD (1800ppm) for 60 min		Washed lettuce	540 ± 141	-	-	-	
NaOCl (100 ppm)+COD (800ppm) for 30 min	Laboratory	Process water	-	-	7.8 ± 1.4	-	(Van Haute et al., 2013b)
NaOCl (150 ppm)+COD (800ppm) for 30 min		Process water	-	-	13.6 ± 2.9	-	
NaOCl (100 ppm)+COD (1500ppm) for 30 min		Process water	-	-	9.3 ± 3.4	-	
NaOCl (150 ppm)+COD (1500ppm) for 30 min		Process water	-	-	13.5 ± 7.8	-	

Table 1.11 (*continued*)

Washing solution	Scale	Samples	Compounds				References
			THMs* µg/g or L	Trichloromethane µg/g or L	Chloroform µg/L	Bromodichlo -romethane	
NaOCl (200 ppm) for 2 min, 50°C	Pilot plant	Process water	-	0.2	-	-	(Klaiber et al., 2005)
NaOCl (200 ppm) for 2 min, 4°C		Process water	-	-	-	-	
NaOCl (200 ppm) for 2 min, 50°C		Washed carrot	-	$2.5\ 10^{-3}$	-	-	
NaOCl (200 ppm) for 2 min, 4°C		Washed carrot	-	-	-	-	
NaOCl maintained 1 ppm+COD (500ppm) for 1h	Laboratory	Washed lettuce	<6.3	-	-	-	(Van Haute et al., 2013b)
NaOCl maintained 1 ppm+COD (500ppm) for 1h		Process water	27.8 ± 5.4	-	-	<6.3	
NaOCl maintained 1 ppm+COD (1000ppm) for 1h		Washed lettuce	<6.3	-	-	-	
NaOCl maintained 1 ppm+COD (1000ppm) for 1h		Process water	124.5 ± 13.4	-	-	13.4 ± 2.9	
NaOCl maintained 5 ppm+ COD (500 ppm) for 60 min	Pilot plant	Process water	1315 ± 8	1299 ± 1	-	-	(Gómez-López et al., 2014)

*THMs: trihalomethanes; **COD: chemical oxygen demand; (-): not determined

1.5.2 Peracetic acid

Peracetic acid (PAA) is a strong oxidant and disinfectant which is one of the most promising and widespread substitutes for chlorine-based disinfectants. Commercial PAA based products are usually quaternary equilibrium mixtures of PAA, hydrogen peroxide (H_2O_2), acetic acid and water as shown in Eq. 4

$$CH_3CO_2H + H_2O_2 \leftrightarrows CH_3CO_3H + H_2O \quad \text{Eq. 4}$$

PAA is a stronger oxidant than chlorine, as illustrated by its higher oxidation-reduction potential of 1.81 eV compared to 1.36 eV for chlorine. The disinfectant activity of PAA is based on the production of active oxygen which can denaturate the proteins and enzymes of microorganisms. The permeability of microorganisms' cell wall will increase by oxidizing sulfhydryl and sulphur bonds in the cell wall proteins and enzymes (Demirci and Ngadi, 2012; Kitis, 2004). The disinfectant efficiency of PAA is high towards bacteria and then decreases in order of magnitude towards viruses, bacterial spores and cysts of protozoa. In addition to its antimicrobial action, the disinfection efficacy of PAA is not affected by fluctuations in pH as is the case for chlorine (Beuchat, 1998; Kitis, 2004). Optimal activity of PAA occurs between pH 3 and 7. Another advantage of PAA over chlorine is that the activity of PAA is less affected by organic matter (Kitis, 2004). Additionally, unlike chlorine, PAA does not react with protein to produce toxic or carcinogenic by products (Silveira et al., 2008) as excessive PAA is broken down to acetic acid and oxygen (Monarca et al., 2002), both of which are safe and environmentally friendly residues (Demirci and Ngadi, 2012). Some studies have also shown that PAA produces harmless by-products of aldehydes and carboxylic acids after oxidation of natural organic matter in water (Kitis, 2004; Monarca et al., 2002). No halogenated disinfection by-products are observed after the treatment of environmental water with PAA (Monarca et al., 2002). In contrast, a major disadvantage of PAA is the higher cost compared to chlorine-based sanitizers. In addition, PAA increases the organic load in processing water to a greater extent than chlorine (Kitis, 2004; López-Gálvez et al., 2009). According to the U.S. Food Drug Administration, a maximum concentration of 80 ppm PAA in the washing water is used for disinfection of fruits and vegetables, and 220 ppm for red meat carcasses and organs (FDA, 2010). To date the use of PAA has been investigated to decontaminate vegetables (Vandekinderen et al., 2009a; Vandekinderen et al., 2009b; Velde et al., 2013) and poultry (Bauermeister et al., 2008). A summary of the studies which have been performed on the decontamination of vegetable products by PAA are shown in **Table**

1.12. Although PAA has been commonly used to treat waste water (Falsanisi et al., 2006; Koivunen and Heinonen-Tanski, 2005) and farmed fish against fish pathogens/disease (Jussila et al., 2011), to date no studies have been reported on the decontamination of fish products in industrial plants.

Table 1.12 Evaluation of PAA on reduction of *E. coli* inoculated on various vegetables

Inoculated Microflora	**Peracetic acid** (ppm)	**Contact time** (min.)	**Log reduction** Log (CFU/g)	**Product**	**References**
E. coli CECT 471, 516 and 533	500	1	2	Cut lettuce	(López-Gálvez et al., 2009)
E. coli O157:H7	40	2	1.24	Shredded carrots	(Ruiz-Cruz et al., 2007)
E. coli O157:H7	60	2	1.07	Cut-wash lettuce	(Palma-Salgado et al., 2014)
E. coli O157:H7	60	2	1.87	Wash-cut lettuce	(Palma-Salgado et al., 2014)
E. coli O157:H7	70	3	2.3	Mung bean sprout	(Neo et al., 2013)

1.6 Concluding remarks and aims of the thesis

Vietnamese *Pangasius* has become appreciated by consumers from different markets all over the world. Besides frozen *Pangasius*, thawed *Pangasius* fillets are commonly marketed in Western countries. Once thawed, the biochemical and microbiological changes of thawed fillets are in principle as fast and similar to those that take place on non-frozen fillets. So far, only one study determined the shelf life under different MAP conditions and identified the dominant microbiota on thawed fillets at the end of shelf life (Noseda et al., 2012). It is reported that *Pseudomonas* and *Serratia* spp. are dominant in Vietnamese *Pangasius* fillets in air condition, *Brochothrix thermosphacta* and *Carnobacterium* spp. in 50% CO_2 + 50% O_2, *B. thermosphacta*, *Serratia* and *Carnobacterium* spp. in 50% CO_2 + 50% N_2 and *Serratia* and *Carnobacterium* spp. in vacuum (**Table 1.5**). Some questions remain concerning the origin of these microorganisms as well as the influence of processing on the microbial evolution and quality. In addition, a review of the RASFF database showed that *Pangasius* originating from Vietnam are sometimes contaminated with pathogenic bacteria such as *Salmonella, L. monocytogenes*, etc. However, knowledge concerning the dynamics of the microbial quality and safety of *Pangasius* processed in Vietnam and intended for export is still very limited.

Lastly, it was evident in literature that whilst the decontamination of fish with chlorine is practiced in some processing companies only a few studies have reported the efficacy of these processes. Most of the decontamination studies done to date have been performed on fresh-cut vegetable products (Allende et al., 2009; Gómez-López et al., 2014; Van Haute et al., 2013a; Van Haute et al., 2013b).

This thesis had the major objective of addressing (partly/as far as possible) these gaps mentioned above. Therefore various studies were performed in this thesis to address:

i) the evolution of microbial quality and safety of *Pangasius* fillets processed industrially in Vietnam and marketed in Belgium.

ii) the dynamics of microbial contaminations was evaluated in *Pangasius* processing lines substantially oriented to export by means of a microbial assessment scheme combined with a self-assessment questionnaire.

and **iii**) the decontamination of *Pangasius* fillets and disinfection of the wash water were studied elaborately at the industrial and laboratory scale.

The research outline is presented in **Figure 1.11**

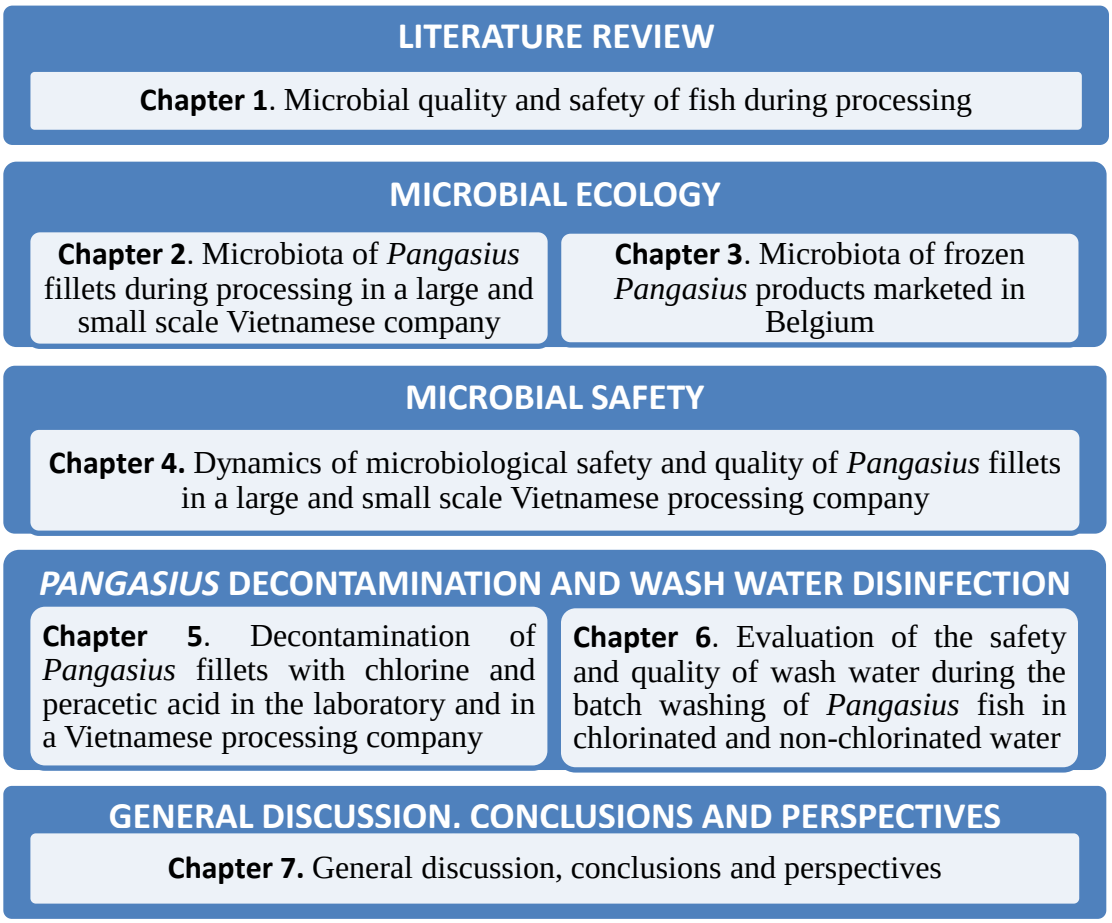


Figure 1.11 Thesis outline

Chapter 2 MICROBIOTA OF *PANGASIUS* FILLETS DURING PROCESSING IN A LARGE AND SMALL VIETNAMESE COMPANY

Redrafted from:

Tong Thi, A.N, Noseda, B., Samapundo, S., Nguyen, B.L., Broekaert, K., Rasschaert, G., Heyndrickx, M., and Devlieghere, F., 2013. Microbial ecology of Vietnamese Tra fish (*Pangasius hypophthalmus*) fillets during processing. International Journal of Food Microbiology. 167(2):144-152.

ABSTRACT

There are numerous factors that can have an impact on the microbial ecology and quality of frozen *Pangasius hypophthalmus* fillets during processing in Vietnam. The presence of spoilage bacteria along the processing line can shorten the shelf-life of thawed frozen fish products. Therefore, the spoilage microbiota throughout the processing chain of two companies (BC: large scale factory, chlorine-based process, BW: large scale factory, water-based process and SC: small scale factory, chlorine-based process) was identified by culture-dependent techniques and 16S rRNA gene sequencing. The microbiological counts were observed to be insignificantly different ($p > 0.05$) between BC and BW. Surprisingly, chlorine water treated fillets from the SC line were revealed to have significantly higher microbial counts than potable water treated fillets at BW line. This was determined to be a result of temperature abuse during processing at SC, with temperatures even greater than 10°C being recorded from skinning onwards. On the contrary, the microbiota related to spoilage for BC and BW lines was determined by 16S rRNA gene sequencing to be more diverse than that on the SC line. A total of 174 isolates, 20 genera and 38 species were identified along the processing chains. The genera *Aeromonas, Acinetobacter, Lactococcus* and *Enterococcus* were prevalent at various processing steps on all the processing lines evaluated. A diverse range of isolates belonging to the *Enterobacteriaceae* such as *Providencia, Shigella, Klebsiella, Enterobacter* and *Wautersiella* were isolated from fillets sampled on the SC line whereas *Serratia* was only observed on fillets sampled on the BC and BW lines. The results can be used to improve Good Manufacturing Practices for processed *Pangasius* fillets and to select effective measures to prolong the shelf-life of thawed Vietnamese *Pangasius* fillets products.

2.1 INTRODUCTION

Tra fish (*Pangasius hypophthalmus*) is a tropical and farmed freshwater fish in Vietnam which is mainly processed into skinless and boneless fillets intended for export to many countries in the world (Karl et al., 2010; Phuong and Oanh, 2010). Although a diverse mixture of bacteria are found in the intestine and the surface of tropical water fish (see § **1.3.1**), the specific microbiota of fresh *Pangasius* is not yet known. During the processing of *Pangasius* fillets, the microorganisms present in the gut and on the skin can spread to the processing equipment, the workers, and sterile flesh fillets. However, the growth of pathogenic and spoilage organisms is retarded by the subsequent freezing applied to frozen *Pangasius* fillet products. Additionally, as these products are cooked before consumption, they appear to be of less risky with regards to microbial foodborne hazards. In addition to frozen products, thawed fillets are widely marketed in Western markets as a (re)fresh product kept at refrigeration temperature. Once thawed, the fish fillets deteriorate primarily through microbiological spoilage (ICMSF, 2005).

Besides bacteria originating from the fish itself, frozen *Pangasius* can be (cross) contaminated with bacteria from working surfaces, equipment, food operators, etc. (Chen et al., 2010; Norton et al., 2001; Rørvik et al., 1995). In practice, chlorinated water is used for disinfection of surfaces, equipment, hands, etc. and for decontamination of fillets during washing in some Vietnamese companies. However, the efficiency of chlorine in reducing microbial levels on *Pangasius* fillets in the washing steps has not yet been confirmed. Moreover, knowledge of the microbial ecology of fish during processing would also provide valuable information for controlling the safety and quality of fish products.

In one of the few existing reports, Noseda et al. (2012) indicated that the dominant spoilage microorganisms of chill-stored, modified atmosphere packaged thawed Vietnamese *Pangasius* fillets were *Serratia*, *Pseudomonas*, *Carnobacterium* and *Brochothrix thermosphacta*. However, to the best of our knowledge, no studies have been done to date describing the evolution of the spoilage microbiota on *Pangasius* fillets during processing.

The major objective of this chapter was to obtain detailed insight into the spoilage microbiota profile of *Pangasius* fillets processed at large and small scale factories in the Mekong Delta region of Vietnam. The microbial ecology of *Pangasius* fillets processed in large and small

factories using chlorinated and/or non-chlorinated water during washing was evaluated. These results give an insight into the dynamic changes of the ecology of the microbiota of Vietnamese *Pangasius* fillets during processing.

2.2 MATERIALS AND METHODS

2.2.1 Processing plants

Two *Pangasius* fillet processing companies located in the Mekong Delta region of Vietnam were evaluated. These two consisted of a large and small scale plant which produce mainly frozen fillet products and have daily production capacities of *ca.* 150 and *ca.* 35 tons, respectively. There are *ca.* 1000 and 300 people working in the large and small scale plant, respectively. Both production plants, were HACCP, BRC and IFS certified. Unlike the small scale company, food safety and/or quality management systems such as ISO 22000:2005 and ISO 9001:2000 were also applied at the large scale company. The *Pangasius* products in the large scale company are largely exported to West European countries (i.e. Belgium, Netherlands, Germany, etc.), the United States, Canada, etc. Those from the small scale company are mainly exported to the European countries (i.e. United Kingdom and Greece), the United Arab Emirates, Egypt, etc.

The small scale plant utilized chlorinated water (SC) as a disinfectant (50 ppm) to decontaminate the fillets during the washing steps. The large scale plant usually did not use chlorinated water (BW), and only utilized chlorine during washing steps as required for certain fish products or at the request of their customers. For purposes of this research, chlorinated water (50 to 80 ppm) was used in washing steps at the large scale plant (BC).

2.2.2 Product manufacturing

The flow charts for the processing of *Pangasius* fillets at the large (BW and BC lines) and small scale (SC line) factories are shown in **Figure 2.1 A** and **B**, respectively. The *Pangasius* fish used at both factories was farmed in different regions of the Mekong Delta (i.e. Dong Thap for the large scale and Can Tho city for the small scale company, respectively).

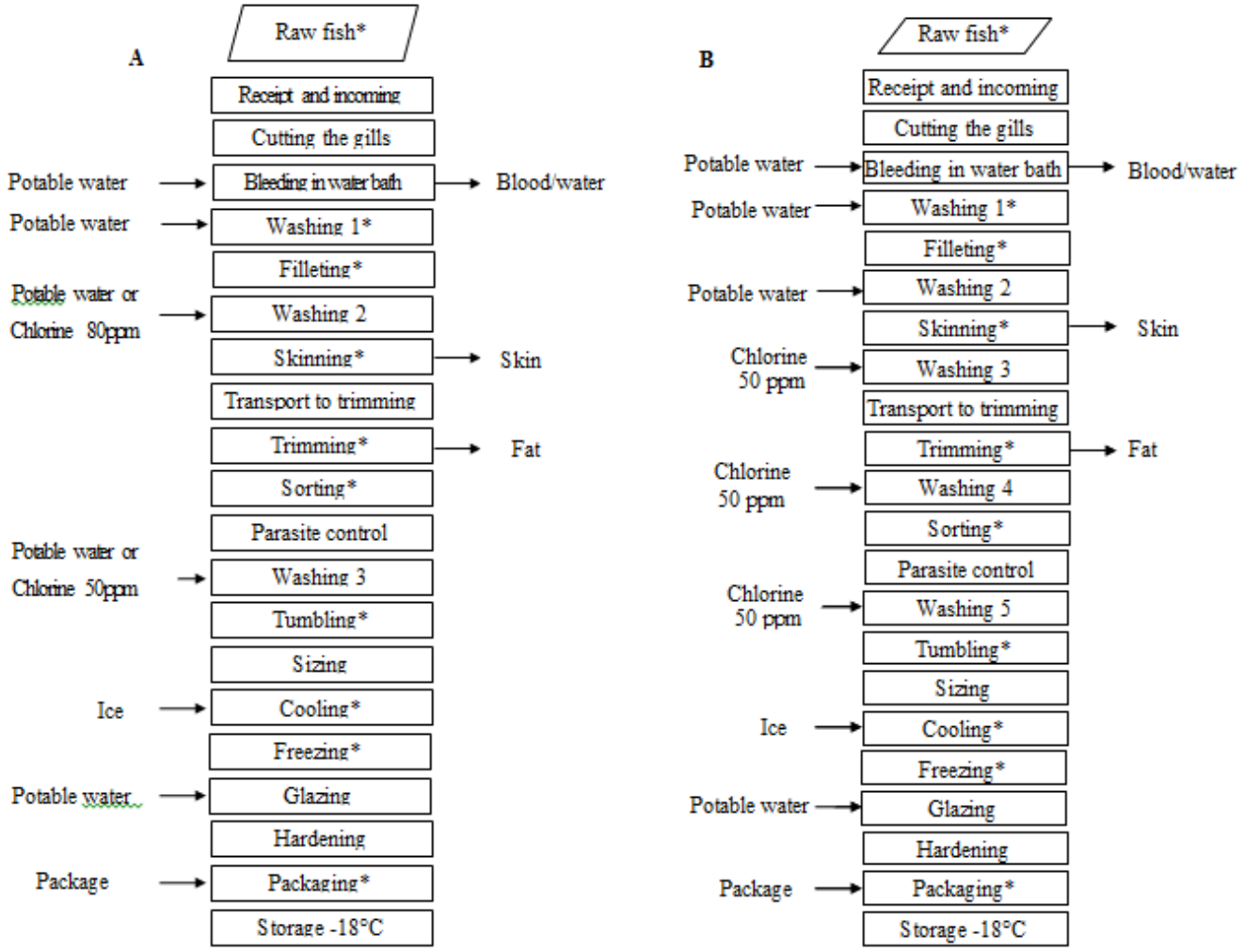


Figure 2.1 Process flow diagram for production of frozen *Pangasius* fillets in A) the large scale plant (chlorine-based process (BC) and water-based process (BW)) and B) the small scale plant chlorine-based process (SC). * Sampling locations

The fish used at the large scale plant were transported alive from farms to the factory in boats (*ca.* 4 hours) whilst the fish for the small scale factory were transported from agencies to factory by van within 30 min. Upon arrival at the companies, bleeding, filleting and washing were done manually followed by mechanical removal of skin by a skinning machine. The fillets were trimmed, sorted and checked putative parasites as was described in § **1.2.2.1** of **Chapter 1**. Next, the fillets were treated with unspecified additives in tumblers for 15 min. in the large scale (BW and BC lines) and for 1 hour in small scale (SC line) factories. The fillets were graded manually by weight and then were cooled with flake ice before freezing. During cooling, five kilograms of the fillets were placed in plastic bags to avoid direct contact with the flake ice. The time of cooling was variable and depended on the availability of freezers. The fillets were then frozen until a core temperature of -18°C. Before packaging and labeling, the frozen fillets were glazed by dipping in cold water mixed with flake ice and frozen a second time. This resulted in fillets with a good appearance (shiny), reduced the rate of fat/lipid oxidation and avoided freezer-burn during storage (Vanhaecke et al., 2010). The packed frozen products were then stored at -18°C in cardboard boxes.

During processing, a surface decontamination process was implemented by washing the fillets intermediately after the main processing steps. There are five washing steps in the large scale company (BC/BW) and three washing steps in the small scale company (SC). In the BW processing line, washing of the fillets was done in a water bath containing a mixture of potable water and flake ice combined with compressed air inflow. In the BC processing line the fillets were washed with a chlorine solution of 80 ppm at washing step 2 and a chlorine solution of 50 ppm at washing step 3 **(Figure 2.1A)**. In BC and BW lines, the wash water was renewed for each washing batch whereas that in the SC processing line was reused for several batches of washing. In the SC processing line, washing was performed with chlorinated water (fixed concentration up to 50 ppm) combined with manual stirring (**Figure 2.1 B**).

2.2.3 Sampling

For each plant, the locations where the samples were collected are indicated in **Figure 2.1A** (BC and BW processing lines) and **Figure 2.1B** (SC processing line) with asterisks. Samples were collected in February 2012 at the large scale plant and in March 2012 at the small scale production plant. At each processing line, samples were collected at three different times in

three consecutive weeks. One fillet (*ca.* 200g) was randomly selected at the end of each processing step designated for sampling. The randomly selected fillets were aseptically taken with sterile tweezers and placed in separate sterile stomacher bags, before sealing was done. Only for the samples of raw fish and samples taken at the washing step 1 (after bleeding), the whole fish was collected. Subsequently, the samples were analyzed in the microbiological laboratory of the large scale company while the packaged samples of the small scale company were then stored in ice and transported in insulated boxes to the Laboratory of Microbiology and Biotechnology of Food Technology Department, Can Tho University, Vietnam for microbiological analyses within 6 to 24 h of sampling.

2.2.4 Microbiological analyses

A 25 g composite sample from different parts of each fillet sample was transferred aseptically to a stomacher bag by means of sterile scalpels and tweezers. 225 ml of sterile Maximum Recovery Diluent (MRD, Merck, Darmstadt, Germany) was added and the mixture was homogenized for 1 min. Further decimal dilutions were prepared in MRD. The total psychrotrophic and mesophilic counts were determined on Plate Count Agar (PCA, Merck, Darmstadt, Germany) by pour plating the decimal dilutions. The pour plates were incubated for 72 h at 22°C and 30°C to determine the total psychrotrophic and mesophilic counts, respectively with the goal of visually selecting as many different colonies as possible for further identification. The number of presumptive *Enterobacteriaceae* spp. were determined by pour plating the decimal dilutions on Violet Red Bile Glucose agar (VRBGA, Merck, Darmstadt, Germany) with an over layer. The VRBGA plates were incubated for 24 h at 37°C after which all colonies were counted. Mesophilic lactic acid bacteria (LAB) were determined by pour plating the decimal dilutions on de Man Rogosa Sharpe agar (MRS, Merck, Darmstadt, Germany) with an over layer followed by incubation for 72 h at 35°C.

2.2.5 Temperature/time measurements

In order to evaluate the processing temperature and time, six measurements were performed on two separate days and at three different moments a day (at 8 a.m., 11 a.m. and 14 p.m.). The temperature inside the processing plants was measured in the filleting (from filleting to skinning step); trimming (from trimming to cooling step) and freezing halls (from freezing to packaging step). The temperature of the fish was also measured after bleeding, filleting,

skinning, before trimming, during trimming, sorting, parasite control, before tumbling, after tumbling, before sizing, sizing, and before freezing. The temperature of all cases was determined by a portable digital thermometer (± 0.1°C) (Multi-thermometer, China). In addition, the transit time of the fillets at these processing steps was measured.

2.2.6 Isolation and identification of dominant microbiota

2.2.6.1 Sample preparation

From each processing line, 25-32 isolates were selected per sampling-day originating from the fillets samples at the beginning (filleting), middle (trimming) and final (freezing) processing steps taking into account as many different colony morphologies (e.g. color, size, and shape) as possible on the media used. A total of 252 isolates were purified and stored on Tryptone Soy Agar (TSA Merck, Darmstadt, Germany) slants at 4°C.

2.2.6.2 DNA-extraction

DNA-extraction was based on a modified protocol Flamm et al. (1984) using lysostaphine (0.5 mg/ml; Sigma) and mutanolysine-lysozyme solution (1 U/ml mutanolysine, Sigma; 2.5 mg/ml lysozyme, Roche) dissolved in HPLC water and TE-buffer [0.05 M Tris, (Invitrogen); 0.02 M EDTA (Merck), pH 8], respectively. These solutions were added to the pellet of the pure culture in the first steps of the DNA-extraction. After extraction, the quality and quantity of DNA were tested by a spectrophotometer (Nanodrop, Isogen) before using as a template DNA.

2.2.6.3 rep-PCR

All isolates were grouped into clusters on the basis of the similarity of their fingerprints obtained with $(GTG)_5$-PCR, which is a rep-PCR technique. The microbial DNA was used as a template in the PCR-reaction. Reactions were carried out in 25 µl volume containing microbial DNA (50 ng/µl), 1x RedGoldstar buffer (75 mM Tris-HCl; Eurogentec) and a final concentration of 3.4 mM of $(GTG)_5$ primer (Eurogentec), 1.5 mM Mg_2Cl (Applied Biosystems), 1 U RedGoldStar DNA polymerase (Eurogentec) and 0.2 mM of each deoxynucleotide triphosphate (GE Healthcare Europe GmbH). Amplification was done in a Geneamp PCR 9700 Thermocycler (Applied Biosystems) using the amplification conditions as follows: initial denaturation at 95°C for 7 min, 30 cycles of 1 min at 94°C, 1 min at 40°C,

8 min at 65°C and a final 16 min extension at 65°C (Versalovic et al., 1994). PCR products were size separated in a 1.5% Seakem LE agarose gel (Lonza) in 1xTBE buffer (0.1 M Tris, 0.1 M Boric acid, 2 mM EDTA) at 120V for 4 h. The $(GTG)_5$ profiles were visualized under UV light after staining with ethidium bromide for 30 min. and a digital image was captured using the G:BOX camera (Syngene). The resulting fingerprints were compared using the Bionumerics version 6.5 software package (Applied Maths, Sint-Martens-Latem, Belgium) using the EZ load 100 bp PCR Molecular Ruler (Biorad) as normalization reference. The similarity between the fingerprints was calculated using the Pearson correlation (1% optimization 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).

2.2.6.4 Identification of the microbial isolates by sequence analysis

A 1500 bp fragment of the 16S rRNA gene was amplified by PCR using forward 16F27 and reserve 16R1522 primers (Brosius et al., 1978). Amplification was performed as follows: initial denaturation at 94°C for 1 min, 25 cycles at 94°C for 15 s, 60°C for 15 s and 72°C for 30 s followed by an elongation step at 72°C for 8 min. 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. The sequence reactions were then performed at Macrogen (Seoul, Korea), using a template of 30-50 ng PCR product DNA and 0.2 µM of primer 16F27 (16S forward primer). The partial 16S rDNA sequences (around 900 bp) was compared with validly published prokaryotic names in the EzTaxon server (http://www.eztaxon.org/; (Chun et al., 2007) to determine the closest phylogenetic relatives of the strains and calculate levels of 16S rDNA gene sequence similarity. A minimum of 98.5% of similarity (unless otherwise indicated) with an EZTaxon entry was used to identify the isolates to the genus level and to the tentative species level. All isolates were additionally characterized by Gram staining, oxidase and catalase test.

2.2.7 Statistical analysis

All experiments were performed in triplicate i.e. on three different fillet samples collected on different days. Results of the microbiological analysis are reported as mean value (log

CFU/g) ± standard deviation of these triplicate analyses. Differences in mean log CFU/g between the processing steps evaluated and between the processing plants were statistically assessed using one way Analysis of Variance (ANOVA) in SPSS version 20 (IBM Inc., Chicago, Ill., USA) when a Shapiro-Wilk test indicated that the means were normally distributed, the validity of which was tested using various normality plots. If a Levene test confirmed heteroscedasticity, a Tamhane's T_2 test was chosen instead of Tukey's test. A non-parametric Kruskal-Wallis H type test was performed in case the data showed non-normality and each comparison with pair means using a Mann-Whitney U test ($\alpha = 0.05$).

2.3 RESULTS

2.3.1 Microbiological profile of Pangasius *fillets during processing*

Evolution of the microbiological counts of Vietnamese *Pangasius* fillets during processing at the different factories is shown in **Table 2.1**. Total psychrotrophic counts of 3.8 ± 0.2, 5.0 ± 0.4 and 4.6 ± 0.6 log CFU/g were found on raw *Pangasius* fish sampled from the BC, BW and SC lines, respectively. These did not differ significantly ($p > 0.05$) from each other. The total mesophilic counts on raw *Pangasius* fish sampled from the BC, BW and SC lines were 4.4 ± 0.6, 5.1 ± 0.5 and 4.7 ± 0.6 log CFU/g, respectively. These did not differ significantly ($p > 0.05$) from each other and from the initial psychrotrophic counts observed on each line. As can be seen in **Table 2.1**, the psychrotrophic and mesophilic counts on the fillets sampled from both the BW and BC lines did not differ significantly ($p > 0.05$) between the processing steps evaluated. In contrast, it can be seen that both the psychrotrophic and mesophilic counts on the fillets sampled on the SC line increased significantly ($p < 0.05$) to *ca*. 5.9 log CFU/g at the trimming, sorting and cooling steps. Thereafter, the psychrotrophic and mesophilic counts on the SC line decreased non-significantly ($p > 0.05$) to 5.1 ± 0.2 log CFU/g.

In addition to the comparison made above of the psychrotrophic and mesophilic counts at each processing step, a comparison was also made between the three processing lines evaluated. It can be seen in **Table 2.1** that no significant differences ($p > 0.05$) occurred in the psychrotrophic and mesophilic counts of the fillets sampled on BC, BW and SC lines from the raw fish up to the filleting steps. Thereafter, from the skinning step onwards, it was observed that psychrotrophic and mesophilic counts on the SC line were significantly higher ($p < 0.05$) than those of the BC and BW lines, with the exception of the freezing step. It was also found that greater variation occurred in both the psychrotrophic and mesophilic counts of

the triplicate samples at the filleting step on the BC and BW lines; with the standard deviations ranging from ± 0.9 to ± 1.1 log CFU/g. This was not observed on the SC line.

In **Table 2.1**, the presumptive *Enterobacteriaceae* counts on raw *Pangasius* fish sampled from the BC, BW and SC lines were 2.5 ± 0.1, 3.9 ± 0.6, and 3.1 ± 1.1 log CFU/g, respectively. These did not differ significantly ($p > 0.05$) from each other. As observed for the psychrotrophic and mesophilic counts, the counts of presumptive *Enterobacteriaceae* on the fillets sampled from both the BW and BC lines was not significantly different ($p > 0.05$) between the processing steps evaluated. In similarity to the trend observed for the psychrotrophic and mesophilic counts on the fillets sampled from the SC line, it was determined that in comparison to the levels on the raw fish, the counts of presumptive *Enterobacteriaceae* on the SC line increased significantly ($p < 0.05$) from the filleting step to a maximum of 5.3 log CFU/g during skinning, trimming, sorting and cooling. Thereafter, the presumptive *Enterobacteriaceae* counts on the fillets sampled from the SC line decreased significantly ($p < 0.05$) by 1 log CFU/g on freezing. It can be further seen in **Table 2.1** that no significant differences ($p > 0.05$) occurred between the presumptive *Enterobacteriaceae* counts of the fillets sampled on BC, BW and SC lines from the raw fish up to the filleting steps. Thereafter, from the skinning step onwards, it was observed that presumptive *Enterobacteriaceae* counts on the SC line were significantly higher ($p < 0.05$) than those of the BC and BW lines.

Generally, the trends observed for the LAB counts were similar to those described above for the total counts and presumptive *Enterobacteriaceae* counts. Namely, no significant differences ($p > 0.05$) occurred between the LAB counts on the fillets sampled at each processing step on both the BC and BW lines. The LAB counts on the fillets sampled from SC line were significantly higher ($p < 0.05$) than those sampled on the BC and BW lines from the filleting step onwards. As observed for the presumptive *Enterobacteriaceae*, freezing reduced LAB counts by *ca*. 1 log CFU/g. In comparison to the total counts and presumptive *Enterobacteriaceae* counts, the LAB counts generally were lower.

Table 2.1. Evolution of the microbiota during processing in the BC: large scale factory-chlorine based process, BW: large scale factory -water based process and SC: small scale factories-chlorine based process.

Medium	Steps	Processing lines		
		BC	BW	SC
PCA psychrotrophic[1]	Raw fish	$3.8 \pm 0.2^{a1*}$	5.0 ± 0.4^{a1}	4.6 ± 0.6^{a1}
	Washing 1	3.7 ± 0.8^{a1}	4.6 ± 0.7^{a1}	5.1 ± 0.6^{ab1}
	Filleting	4.2 ± 0.9^{a1}	3.8 ± 1.1^{a1}	5.1 ± 0.1^{ab1}
	Skinning	3.4 ± 0.3^{a1}	3.9 ± 0.5^{a1}	5.6 ± 0.5^{ab2}
	Trimming	3.8 ± 0.6^{a1}	3.5 ± 0.3^{a1}	5.9 ± 0.1^{b2}
	Sorting	3.2 ± 0.5^{a1}	3.4 ± 0.1^{a1}	5.9 ± 0.1^{b2}
	Tumbling	3.6 ± 0.2^{a1}	3.8 ± 0.5^{a1}	5.3 ± 0.5^{ab2}
	Cooling	3.8 ± 0.6^{a1}	3.8 ± 0.3^{a1}	5.8 ± 0.3^{b2}
	Freezing	3.8 ± 0.7^{a1}	4.1 ± 0.1^{a1}	5.0 ± 0.4^{ab1}
	Packaging	3.8 ± 0.2^{a1}	4.3 ± 0.5^{a12}	5.1 ± 0.2^{ab2}
PCA mesophilic[2]	Raw fish	4.4 ± 0.6^{a1}	5.1 ± 0.5^{a1}	4.7 ± 0.6^{a1}
	Washing 1	3.7 ± 0.8^{a1}	4.7 ± 0.7^{a1}	5.0 ± 0.3^{ab1}
	Filleting	4.2 ± 1.0^{a1}	4.4 ± 1.1^{a1}	5.2 ± 0.1^{ab1}
	Skinning	3.4 ± 0.4^{a1}	4.3 ± 0.5^{a1}	5.7 ± 0.5^{ab2}
	Trimming	3.8 ± 0.7^{a1}	3.9 ± 0.3^{a1}	5.9 ± 0.2^{b2}
	Sorting	3.1 ± 0.4^{a1}	3.5 ± 0.0^{a1}	5.9 ± 0.1^{b2}
	Tumbling	3.8 ± 0.2^{a1}	3.9 ± 0.7^{a1}	5.2 ± 0.5^{ab2}
	Cooling	3.5 ± 0.2^{a1}	3.6 ± 0.2^{a1}	5.8 ± 0.2^{b2}
	Freezing	3.9 ± 0.8^{a1}	4.3 ± 0.3^{a1}	5.0 ± 0.4^{ab1}
	Packaging	3.7 ± 0.5^{a1}	4.4 ± 0.5^{a12}	5.1 ± 0.2^{ab2}
VRBGA[3]	Raw fish	2.5 ± 0.1^{a1}	3.9 ± 0.6^{a1}	3.1 ± 1.1^{a1}
	Washing 1	2.4 ± 0.6^{a1}	2.9 ± 0.8^{a1}	4.4 ± 0.4^{abc1}
	Filleting	3.5 ± 1.5^{a1}	2.4 ± 1.5^{a1}	4.6 ± 0.3^{b1}
	Skinning	2.4 ± 0.8^{a1}	3.3 ± 0.6^{a1}	5.3 ± 0.5^{c2}
	Trimming	2.1 ± 0.7^{a1}	2.6 ± 0.3^{a1}	5.3 ± 0.0^{c2}
	Sorting	1.6 ± 0.8^{a1}	2.6 ± 0.2^{a1}	5.3 ± 0.3^{c2}
	Tumbling	2.7 ± 0.2^{a1}	3.0 ± 0.4^{a1}	4.8 ± 0.7^{abc2}
	Cooling	2.8 ± 0.3^{a1}	2.8 ± 0.4^{a1}	5.3 ± 0.2^{c2}
	Freezing	2.4 ± 0.3^{a1}	2.8 ± 0.0^{a1}	4.3 ± 0.6^{ab2}
	Packaging	2.3 ± 0.3^{a1}	2.7 ± 0.1^{a1}	4.3 ± 0.1^{ab2}
MRS	Raw fish	1.1 ± 0.1^{a1}	$<1.0^{ab1}$	$1,9 \pm 0.8^{a1}$
	Washing 1	$<1.0^{a1}$	1.1 ± 0.1^{a1}	2.3 ± 0.3^{a1}
	Filleting	1.2 ± 0.2^{a1}	1.2 ± 0.2^{ab1}	3.1 ± 0.5^{ab2}
	Skinning	$<1.0^{a1}$	1.1 ± 0.5^{a1}	2.5 ± 0.3^{a2}
	Trimming	1.2 ± 0.6^{a1}	$<1.0^{a1}$	3.0 ± 0.3^{ab2}
	Sorting	1.2 ± 0.4^{a1}	1.8 ± 0.3^{b1}	3.4 ± 0.5^{ab2}
	Tumbling	1.5 ± 0.4^{a1}	1.6 ± 0.6^{ab1}	3.1 ± 0.7^{ab2}
	Cooling	1.2 ± 0.2^{a1}	2.0 ± 0.2^{ab1}	4.2 ± 0.2^{b2}
	Freezing	1.5 ± 0.1^{a1}	1.7 ± 0.3^{b12}	3.0 ± 0.9^{ab2}
	Packaging	1.5 ± 0.3^{a1}	1.9 ± 0.0^{b1}	3.4 ± 0.4^{ab2}

*Data are expressed as mean value ± standard deviation (log CFU/g) of three replicates. Value with a different superscript letter between processing steps in the same column show statistical significance. Value with a different superscript number between companies in the same row show statistical significance ($p < 0.05$)

[1] incubated at 22°C; [2] incubated at 30°C; [3]all colonies on the plates were counted

2.3.2 Temperature/time evolution

2.3.2.1 Processing halls

Inside the large scale factory (BC and BW lines), the air conditioning maintained temperatures of 18.5 ± 0.7°C, 19.0 ± 0.9°C and 20.9 ± 1.1°C in the freezing, trimming and filleting halls, respectively. In contrast, the temperatures in processing halls of the small scale factory (SC line) was higher, being 22.0 ± 0.8°C, 25.4 ± 0.2°C and 25.7 ± 0.7°C in the freezing, trimming and filleting halls, respectively.

2.3.2.2 Pangasius fillets

Evolution of the temperature of the fillets can be seen in **Figure 2.2.** The temperature of the fish at the start of processing (after bleeding) on the BC and BW lines was 29 ± 0.2°C, this temperature decreased steadily to 10.6 ± 0.6°C at the parasite control step. Thereafter the temperature remained unchanged up to the sizing step, after which it decreased to 7.2 ± 1.1°C during preparation for freezing. The fillets sampled on the SC line generally had higher temperatures than those sampled on the BC and BW lines. Three distinct stages can be observed i) from bleeding to the trimming step where the fillets had a temperature from 28.5 ± 1.3°C to 30.6 ± 0.4°C ii) from sorting to tumbling where the fillets had a temperature from 24.6 ± 2.4°C to 25.1 ± 1.5°C and iii) from tumbling onwards where the fillets had a temperature from 18.5 ± 1.5°C to 19.4 ± 1.5°C.

Besides temperature, the transit time of the fish through the production process was also recorded and is shown in **Figure 2.2**. The transit time at each step evaluated was generally longer at the large scale factory (BC and BW lines) than at the small scale factory (SC lines). For example, the transit time during sorting took 6.3 ± 0.6 min and 1.0 ± 0.5 min at the large and small scale plants, respectively. However, some exceptions occurred i.e. the transit time at the skinning step took 1.7 ± 0.6 min and 3.7 ± 0.6 min at the large and the small scale factory, respectively.

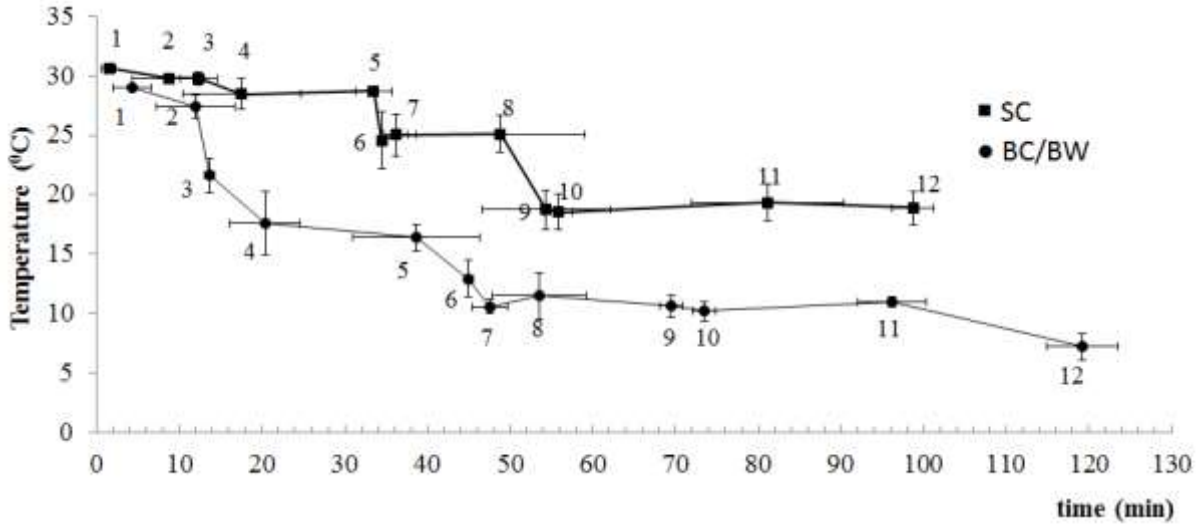


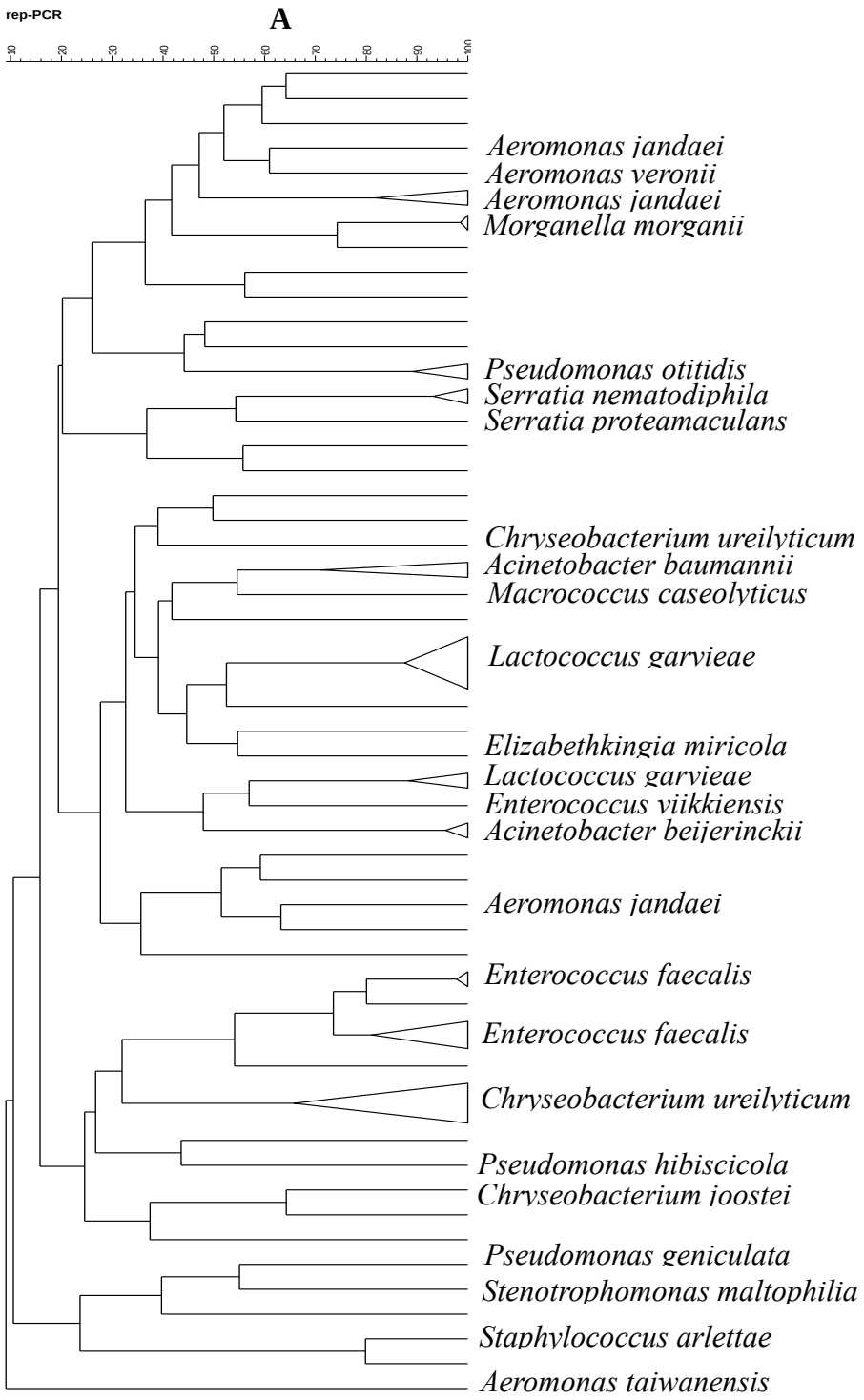
Figure 2.2. Temperature and time evolution of fillets during processing in the large scale plant (BC/ BW) and small scale plant (SC). Error bars denote standard deviation of temperature (X-axis) and time (Y-axis). (1) After bleeding bath; (2) Filleting; (3) Skinning; (4) Before trimming; (5) During trimming; (6) Sorting; (7) Parasite control; (8) Before tumbling; (9) After tumbling; (10) Before sizing; (11) Sizing; (12) Before freezing.

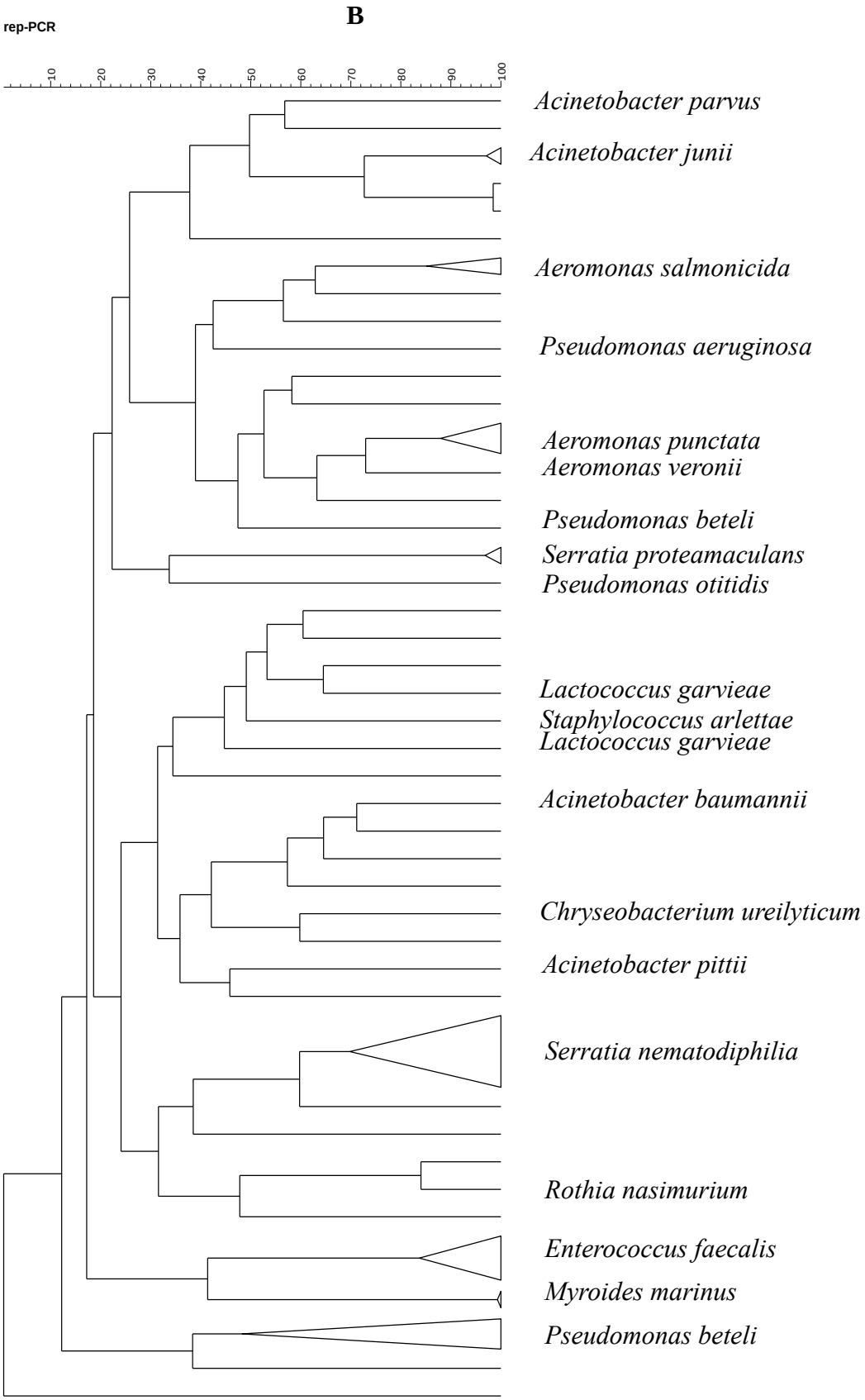
2.3.3 Identification of the isolates recovered at the different processing steps

A total of 252 isolates were selected from the PCA, VRBGA and MRS plates originating from *Pangasius* fillets sampled during the filleting, trimming and freezing steps on the three processing lines. These processing steps represented the beginning, intermediate and final steps of the production line. These isolates were then grouped into clusters based on the visual similarity of their $(GTG)_5$–PCR fingerprints. Dendrograms with the grouping of isolates for each line separately are shown in **Figure 2.3 A, B, C** for large scale factory-chlorine based process-BC, large scale factory-water based process-BW, and small scale factory-chlorine based process-SC, respectively. Two representatives at both edges from each cluster containing at least four isolates and showing a similarity level of at least 65% were selected. Some identified isolates appear as single isolates in a separate clustering per production line, but were grouped on the basis of visually similar fingerprints with other isolates in a global clustering of all three production lines. For clusters with less than four isolates, a single isolate was selected for further analysis by 16S rRNA gene sequencing. The tentative species identification of the selected isolates (on the basis of the EzTaxon database) was extrapolated for the entire group of isolates in each cluster.

A total of 174 isolates were identified. Seventy-eight isolates, which remained single in the global $(GTG)_5$-PCR clustering of the three production lines and probably partly represented minor species of the *Pangasius* microbiota or other genetically different strains of the identified species, were not further identified. The identified isolates consisted of 20 different genera and 38 different species of which 131 isolates were Gram-negative and 43 isolates were Gram-positive (**Table 2.2**). On the basis of the isolates identified, *Aeromonas* spp. (32/174 isolates or 18.4% of the isolates), *Acinetobacter* spp. (19.5%), *Lactococcus* spp. (13.8%) and *Enterococcus* spp. (8%) occurred the most frequently at both plants.

Isolates from the genera *Vagococcus*, *Providencia*, *Shigella*, *Klebsiella* were only found on fillets sampled from the SC line. *Pseudomonas* spp. and *Serratia* spp. were only found in *Pangasius* fillets sampled from the BC and BW lines. Whilst the *Pangasius* fillets sampled from the large scale plant had a similar diversity of bacteria on both lines (BC and BW), exceptions were *Myroides* spp. and *Rothia* spp. which were only isolated from fillets from the BW line.


rep-PCR
A
10
20
30
40
50
60
70
80
90
100
Aeromonas jandaei
Aeromonas veronii
Aeromonas jandaei
Morganella morganii
Pseudomonas otitidis
Serratia nematodiphila
Serratia proteamaculans
Chryseobacterium ureilyticum
Acinetobacter baumannii
Macrococcus caseolyticus
Lactococcus garvieae
Elizabethkingia miricola
Lactococcus garvieae
Enterococcus viikkiensis
Acinetobacter beijerinckii
Aeromonas jandaei
Enterococcus faecalis
Enterococcus faecalis
Chryseobacterium ureilyticum
Pseudomonas hibiscicola
Chryseobacterium joostei
Pseudomonas geniculata
Stenotrophomonas maltophilia
Staphylococcus arlettae
Aeromonas taiwanensis


B
rep-PCR
10
20
30
40
50
60
70
80
90
100
Acinetobacter parvus
Acinetobacter junii
Aeromonas salmonicida
Pseudomonas aeruginosa
Aeromonas punctata
Aeromonas veronii
Pseudomonas beteli
Serratia proteamaculans
Pseudomonas otitidis
Lactococcus garvieae
Staphylococcus arlettae
Lactococcus garvieae
Acinetobacter baumannii
Chryseobacterium ureilyticum
Acinetobacter pittii
Serratia nematodiphilia
Rothia nasimurium
Enterococcus faecalis
Myroides marinus
Pseudomonas beteli

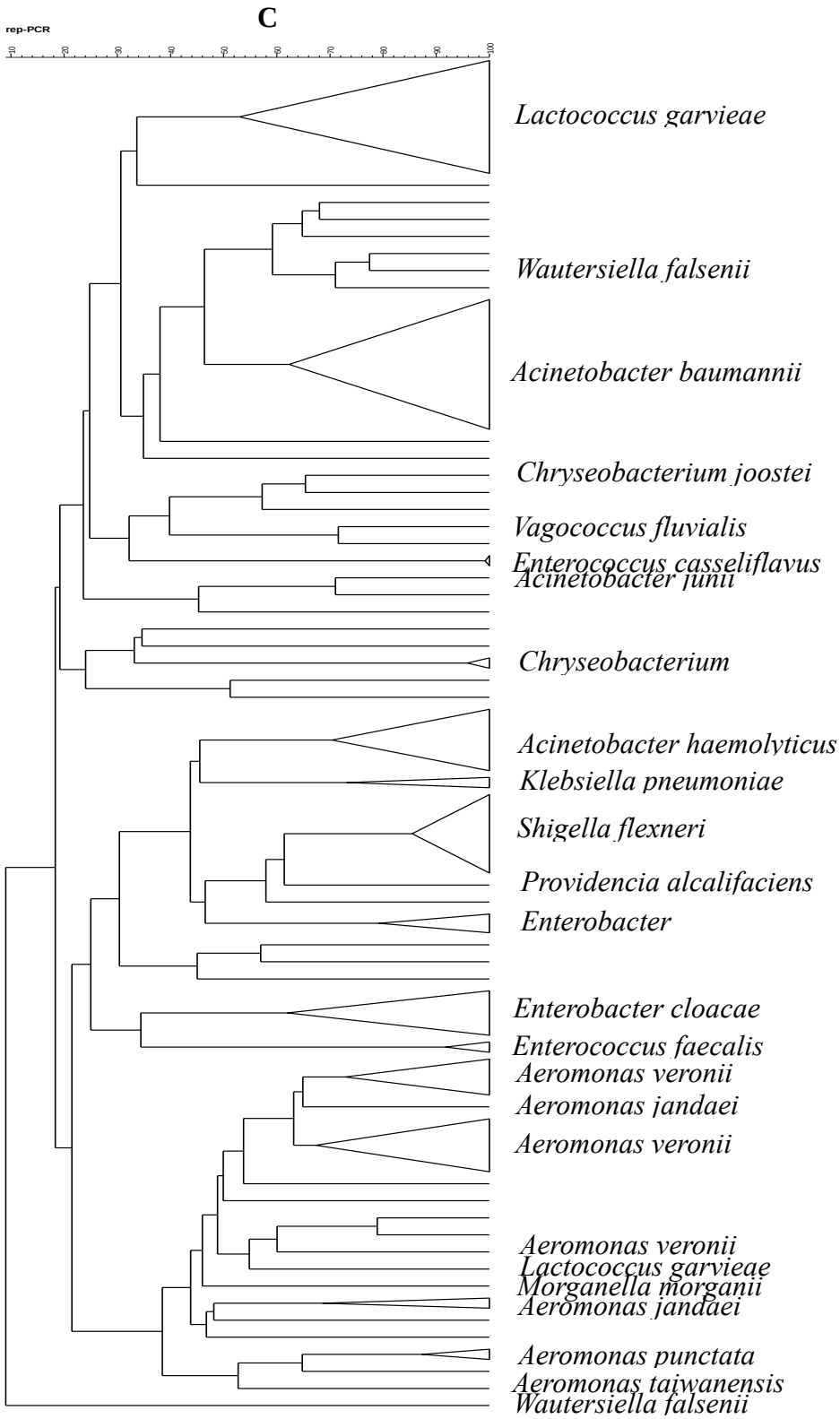


Figure 2.3 Dendrograms generated after cluster analysis of the $(GTG)_5$-PCR fingerprints from 252 isolates (A) large scale factory-chlorine based process-BC, (B) large scale factory-water based process-BW, and (C) small scale factory -chlorine based process-SC

Table 2.2 Genera and species isolated from the fillets throughout process at the filleting, trimming and freezing steps in three processing lines

Identification[1]	Filleting			Trimming			Freezing			Total isolates	Prevalence (%)
	BC	BW	SC	BC	BW	SC	BC	BW	SC		
Aeromonas	**5**[2]	**3**	**10**		**4**	**5**	**1**		**4**	**32**	18.4
Aeromonas salmonicida		2									
Aeromonas veronii	1	1	6		1	3			4		
Aeromonas jandaei	3		3				1				
Aeromonas punctata					3	2					
Aeromonas taiwanensis	1		1								
Acinetobacter			**5**	**2**	**2**	**7**	**2**	**3**	**13**	**34**	19.5
Acinetobacter baumannii			5	2	1	7			4		
Acinetobacter pittii								1			
Acinetobacter junii								2	1		
Acinetobacter beijerinckii							2				
Acinetobacter haemolyticus									8		
Acinetobacter parvus					1						
Pseudomonas	**1**	**1**		**3**	**5**					**10**	5.7
Pseudomonas otitidis				2	1						
Pseudomonas hibiscicola				1							
Pseudomonas geniculata	1										
Pseudomonas beteli		1			3						
Pseudomonas aeruginosa					1						
Stenotrophomonas				**1**						**1**	0.6
S. maltophilia				1							
Serratia							**3**	**8**		**11**	6.3
Serratia nematodiphila							2	6			
Serratia proteamaculans[*]							1	2			
Enterobacter						**6**			**3**	**9**	5.2
Enterobacter cloacae						5			1		
Enterobacter cancerogenus						1			2		
Providencia			**1**							**1**	0.6
Providencia alcalifaciens			1								
Shigella						**9**			**3**	**12**	6.9
Shigella flexneri						9			3		
Klebsiella						**1**			**1**	**2**	1.1
Klebsiella pneumoniae						1			1		
Morganella	**2**		**1**							**3**	1.7
Morganella morganii	2		1								
Wautersiella						**1**			**1**	**2**	1.1
Wautersiella falsenii						1			1		
Chryseobacterium	**4**		**2**		**1**	**1**	**2**	**1**		**11**	6.3
C. joostei[*]						1	1				
C.ureilyticum[*]	4		2		1		1	1			
Myroides								**2**		**2**	1.1
Myroides marinus								2			
Elizabethkingia							**1**			**1**	0.6
Elizabethkingia miricola							1				
Total Gram-negative	**12**	**4**	**19**	**6**	**12**	**30**	**9**	**14**	**25**	**131**	**75.3**

Table 2.2 *(continued)*

Identification	Filleting			Trimming			Freezing			Total isolates	Prevalence (%)
	BC	BW	SC	BC	BW	SC	BC	BW	SC		
Lactococcus	**5**		**5**	**1**	**1**	**4**	**1**	**1**	**6**	**24**	13.8
Lactococcus garvieae	5		5	1	1	4	1	1	6		
Enterococcus		**1**		**2**			**4**	**3**	**4**	**14**	8
Enterococcus faecalis		1		2			3	3	2		
Enterococcus viikkiensis							1				
Enterococcus casseliflavus									2		
Vagococcus			**1**							**1**	0.6
Vagococcus fluvialis			1								
Macrococcus	**1**									**1**	0.6
Macrococcus caseolyticus	1										
Staphylococcus	**1**							**1**		**2**	1.1
Staphylococcus arlettae	1							1			
Rothia					**1**					**1**	0.6
Rothia nasimurium[*]					1						
Total Gram-positive	**7**	**1**	**6**	**3**	**2**	**4**	**5**	**5**	**10**	**43**	**24.7**
Total identified isolates	19	5	25	9	14	34	14	19	35	174	
Number of species	9	4	9	6	10	10	10	9	12		

BC: large scale factory-chlorine based process, BW: large scale factory -water based process and SC: small scale factory -chlorine based process

[1]Identification result on genus and species level; species identifications are only tentative

[2]The frequency of identified isolates based on partial 16S rRNA gene sequence analysis with cut-off value of 98.5% similarity with type strains of validly published prokaryotic names in EZTaxon database. The percentage of total number of isolates (or the prevalence in %) of each genus is listed in the last column.

[*]Some strains identified to the tentative species level had a lower cut-off value than 98.5% similarity (but higher than 97% similarity) with a valid species, this was the case for *Chryseobacterium ureilyticum* (97.5%), *Chryseobacterium joostei* (97.6%), *Serratia proteamaculans* (98%) and *Rothia nasimurium* (98%).

2.4 DISCUSSION

2.4.1 Evolution of microbiological ecology during the processing of Pangasius *fish*

The total mesophilic and psychrotrophic counts on *Pangasius* fillets did not differ significantly ($p > 0.05$) on all three processing lines evaluated. Previous studies have indicated that mesophilic microorganisms are dominant on tropical fish (Gram and Huss, 1996). Ercolini et al. (2009) have reported that whilst mesophilic bacteria (isolated from refrigerated meat) grew fast at both 30°C and 20°C, their psychrotrophic counterparts grew slowly or did not grow at all at 30°C. Therefore, despite the non-significant difference

between the total mesophilic and psychrotrophic counts, it can be deduced that the mesophilic bacteria was dominant on the *Pangasius* fillets sampled.

The total psychrotrophic counts on the frozen fillets after final packaging were 3.8 ± 0.2 log CFU/g (BC), 4.3 ± 0.5 log CFU/g (BW) and 5.1 ± 0.2 log CFU/g (SC). These counts were in good agreement with those reported by Noseda et al. (2012) for thawed Vietnamese *Pangasius* fillets. These counts are within the acceptable limits of the official standard established by Vietnamese Science & Technology Ministry (TCVN, 2010) and the guidelines for fresh fish after production established by the Laboratory of Food Microbiology and Food Preservation (LFMFP) (Ghent, Belgium) (Uyttendaele et al., 2010).

Enterobacteriaceae and LAB have been used to assess hygienic practices in industrial production plants, particularly in meat and fish processing units (Audenaert et al., 2010; Bagge-Ravn et al., 2003; Lebert et al., 2007). *Enterobacteriaceae* on the SC line increased significantly ($p < 0.05$) during the filleting step. The LAB counts on the fillets sampled from SC line increased during processing and increased significantly ($p < 0.05$) in cooling step. The endogenous microorganisms from the gills or intestinal tracts of the fish can contaminate the bacteria present on the flesh during filleting (Ringø et al., 2006; Vijayabaskar and Somasundaram, 2008; Yang et al., 2007). Some strains of *Enterobacteriaceae* such as *Klebsiella pneumoniae, Enterobacter aerogenes* and *Escherichia coli* have been isolated from the intestines of tropical freshwater fish (Apun et al., 1999). Moreover, gut samples from the fillets collected on the BC and BW lines revealed high total psychrotrophic counts of 6.0 ± 0.8 log CFU/g and presumptive *Enterobacteriaceae* counts of 4.2 ± 0.1 log CFU/g (data not shown). In addition, the gut perforation can occur during manual filleting by knives. As a result of this, greater variation was observed between the total mesophilic and psychrotrophic counts (standard deviation = ± 1 log CFU/g) and the presumptive *Enterobacteriaceae* (standard deviation = ± 1.5 log CFU/g) of the three fillets sampled at the filleting step on the BC and BW lines.

Furthermore, as trimming and sorting were done manually, cross contamination can occur at these steps from the food contact surfaces such as gloves, plates, knives, tables, baskets etc. Microorganisms are able to attach to food contact surfaces (Aarnisalo et al., 2006; Fonnesbech Vogel et al., 2001) and some can survive on them even after cleaning and disinfection (Bagge-Ravn et al., 2003). These can detach at a later stage and transfer to the

food product during processing (Kumar and Anand, 1998). The companies evaluated in this chapter have implemented the HACCP principles and Good Manufacturing Practices (GMP). However, some previous studies have indicated that the performance of food safety management systems can vary (Cormier et al., 2007; Higuera-Ciapara and Noriega-Orozco, 2000). In this study cleaning and disinfection of the contact surfaces at the trimming step was done approximately every five hours on the SC line while this was performed every two hours on the BC and BW lines during visits.

The fillets sampled on the small scale (SC) line generally had much higher temperatures than those sampled in the large scale plant (BC and BW lines) (**Figure 2.2**). It is recommended to hold raw fish at <10°C throughout processing to inhibit the growth and toxin production of pathogenic bacteria (FDA, 2011). However, based on the recorded temperatures, the temperature of the fillets sampled on the BC and BW lines decreased steadily with each processing step while those from the SC line experienced mild temperature abuse. Differences were observed in the production processes of the two companies evaluated which could explain these differences. For instance, during visit, no ice was used on the SC line to cool the fillets with the exception of the cooling step. In contrast an over layer of ice (*ca.* 1:5, ice:fish) was placed on the fillets from the skinning step onwards on the BC and BW lines of the large scale plant. The differences in the temperature of fillets sampled at the two companies could have also contributed to the higher bacterial counts observed on the *Pangasius* fillets sampled from the SC line.

Generally, the transit time of the fillets at each processing step evaluated was longer on the BC and BW lines than it was on the SC line. The total transit time from raw fish to before freezing step was *ca*. 100 min on the SC line and *ca.* 120 min on the BC and BW lines. This excludes the time taken during the washing steps, tumbling and cooling. The processing time can vary greatly depending on numerous factors such as the product requirements, demand and production capacity, competence of the workers etc.

Chlorine, a disinfection agent, is essentially used to ensure water safety, hygiene of food contact surfaces and prevention of cross contamination (FAO/WHO, 2008). In addition, in fishery processing, chlorine-based decontamination is widely implemented to reduce microbial loads and to develop a lighter fish or squid color during storage (Benjakul et al., 2012; Kim et al., 1999b). One of the aims of this chapter was to assess the effectiveness of chlorine-based decontamination (BC and SC lines) compared to washing with potable water

(BW line) on reducing the microbial load in industrial practices. However, as mentioned earlier in the large company, no significant difference ($p > 0.05$) occurred in the number of microorganisms investigated during processing with chlorinated and non-chlorinated washing water on the BC and BW, respectively. On the other hand, chlorination was implemented on the SC line, where significantly higher microbial counts ($p < 0.05$) were generally observed compared to those on the fillets from both the BW and BC lines.

On the SC and BC lines, the producers added commercial sodium hypochlorite to potable water at a certain concentration (**Figure 2.1A & B**). This solution consists of the unionized form of hypochlorous acid (HOCl) due to hydrolysis. Next, hypochlorous acid will dissociate to the hypochlorite ion (ClO^-) and proton (H^+) depending on the pH of the water (Fukuzaki, 2006). The effective bactericidal effect of chlorine depends on the stability and activity of free chlorine (as HOCl), which can be overlooked by producers. The pH of the chlorine solutions should ideally be maintained between 6.5 and 7.5 during washing (Suslow, 2008). However, the pH value of the chlorine solutions was not adjusted at both the BC or SC lines. Chlorine may be more a hygienic aid for the fillet surfaces rather than a decontamination tool since some earlier studies emphasized that bactericidal action of chlorine was affected by available chlorine in the wash-water, pH, temperature, exposed time and organic matter present (Suslow, 2008). The microbiological quality throughout the processing chain appeared to be independent of the use of chlorine in the wash water. Therefore, processors should pay more attention to ensure the proper use of chlorine in the conditions it is used during the processing of *Pangasius*. In addition, the should take this into consideration and consider other factors namely quality of materials, equipment, facilities, sanitation program, personal hygiene, temperature control etc. (Jacxsens et al., 2009b) to improve the microbial quality of their products instead of using chlorine. Additionally, the use of chlorinated water for decontamination is currently not allowed in the EU for export purposes (EU, 2004b).

2.4.2 Identification of the spoilage related microbiota

A majority of Gram-negative bacteria (131/174 isolates) were identified using partial 16S rDNA sequence. This fact confirms previous findings that Gram-negative bacteria are typically dominant in flesh fish (González-Rodríguez et al., 2002; Leroi et al., 1998; Paludan-Müller et al., 1998). Fillet samples from the large company (BC and BW lines) generally had a higher diversity of species than those from the small company (SC line). This could have

been a result of differences i.e. in the location (environment) of the companies (the small company was located in Can Tho whilst the large company was located in Dong Thap city), source of water, suppliers (fish farms) and production capacity. In addition, the differences in environmental temperature between two companies sampled could have greatly affected the species.

Aeromonas spp., *Acinetobacter* spp., *Lactococcus* spp., and *Enterococcus* spp., are all known spoilage bacteria on freshwater fish (González et al., 2001; Gram and Huss, 1996; ICMSF, 2005). Some *Acinetobacter* spp. such as *A. baumannii* are also known as opportunistic human pathogens (Giamarellou et al., 2008). These genera were abundant on the *Pangasius* fillets collected at the filleting, trimming and freezing steps. *Aeromonas* spp. and *Acinetobacter* spp. are known to be present in gastrointestinal tract, gills and on surface of the flesh of farm raised freshwater fish (Austin, 2002; Hatha, 2002; Radu et al., 2003; Vivekanandhan et al., 2005). Some strains of *Aeromonas* spp. such as *A. salmonicida* are also known as fish pathogens (Cabello, 2006). Additionally, the LAB (*Lactococcus* spp. and *Enterococcus* spp.) found on the fillets in this chapter have been associated with aquatic environments or infected humans (Kusuda and Salati, 1999; Michel et al., 2007). In particular, one *Lactococcus* strain was determined to be *Lactococcus garvieae,* which is a known fish pathogen (Vendrell et al., 2006). These contaminating bacteria can therefore originate from materials, food operators or the environment throughout the processing chain. However, these species may have been over represented in this chapter due to their capability to grow not only on non-selective media used i.e. PCA, but also on selective or specific media including VRBGA and MRS.

Enterobacteriaceae, namely *Providencia, Shigella, Klebsiella, Enterobacter, Morganella,* and *Serratia,* which can affect safety and shelf-life of *Pangasius* products, were isolated from the *Pangasius* fillets sampled in this chapter. This finding partly explains the origin of *Serratia* spp., which were determined by Noseda et al. (2012) to be the dominant spoilage flora of Vietnamese *Pangasius* fillets stored in air or in MAP condition. It implies that *Serratia* spp. (11/174 isolates) isolated from the BC/BW lines are present on frozen Vietnamese *Pangasius* products before exporting. In difference to the fillets sampled on the BC and BW lines, *Serratia* spp. appeared to be less common on the fillets sampled from the small scaled company (SC line) while various other genera of *Enterobacteriaceae* were only identified at this company such as *Providencia* spp., *Shigella* spp., *Klebsiella* spp. and *Enterobacter* spp. These genera have been isolated from the digestive tracts of freshwater fish

(Austin, 2002; Yagoub, 2009). Previously, Lampel et al. (1999) and Lopez-Sabater et al. (1994) have also reported that these genera can occur as a result of unhygienic handling and poor personal hygiene. Although the focus was on spoilage bacteria, the human pathogen *Shigella flexneri* (12/174 isolates) was detected on some fillets sampled at final processing step. The source of *S. flexneri* contamination in the final products could be the fish harvested from feacally contaminated water, the use of unsanitary water in processing or from food handlers. The presence of *S. flexneri* may be a potential hazard due to its low infectious dose, which ranges from 10 to 100 CFU (Tham and Danielsson-Tham, 2013).

Pseudomonas spp. e.g. *P. otitidis*, *P. hibiscicola*, *P. geniculata*, *P. beteli* and *P. aeruginosa* were identified on fillets at the filleting and trimming steps on both the BC and BW lines. Several studies have shown that *Pseudomonas* spp., which are environmentally opportunistic bacteria, can resist sanitizers and disinfectants used in processing plants (Bagge-Ravn et al., 2003). As a result, their adherence on contact surfaces can lead to the contamination of *Pangasius* fillets during processing. *Pseudomonas* spp. are commonly found in tropical freshwater and are known to be specific spoilage microorganisms of iced fresh water fish in general (Ghaly et al., 2010; Gram, 1993; Gram and Dalgaard, 2002; Gram and Melchiorsen, 1996; ICMSF, 2005). In addition, *Pseudomonas* spp. have been known to be amongst the dominant organisms present on thawed Vietnamese *Pangasius* products at the end of their shelf-life (Noseda et al., 2012). However, in difference to Noseda et al. (2012) who isolated *Pseudomonas* spp. from frozen *Pangasius* products from Vietnam, no *Pseudomonas* spp. were found on frozen fillets after the freezing step in this study. It is possible that *Pseudomonas* isolates belonged to the minor microbiota (represented as single isolates in the global $(GTG)_5$-PCR dendrogram) on frozen fillets.

Not surprisingly, the diversity of identified species did not differ greatly between fillets sampled from the BC and BW lines. Few exceptions were for *Myroides marinus* and a *Rothia nasimurium* isolate, which were only present on the BW line. *Rothia nasimurium* is a member of the family *Micrococcaceae*. *Myroides* spp. are widely distributed in the aquatic environments and have also been isolated from biofilm structures in food processing plants (Jacobs and Chenia, 2009; Vishnivetskaya et al., 2009).

Chyseobacterium including *C. jooste* and *C. ureilyticum* (11/174) were found throughout the processing steps on fillets sampled from all three processing lines. *Chryseobacterium* spp.

and *Elizabethkingia* spp., belonging to the family *Flavobacteriaceae*, have been isolated from unhealthy fish, water (even in chlorine-treated water), soil and aquatic environments (De Beer et al., 2006; Ilardi et al., 2009; Olofsson et al., 2007; Zamora et al., 2012). However, to our knowledge, only Ramos and Lyon (2000) have emphasized the role of *Chryseobacterium* spp. as spoilage bacteria in catfish products.

2.5 CONCLUSION

The ecology of the spoilage microbiota of Vietnamese *Pangasius* fillets during its processing from raw materials to final products was determined by conventional and molecular techniques. In general, the microbiological counts during processing were determined to be dependent on the factories but independent of the use of chlorinated water during the washing of fillets. Besides the total microbiological counts, the high presumptive counts and the diversity of species of *Enterobacteriaceae* spp. is likely to reflect a potential hazard of microbiological food safety at the small scale factory. Therefore, future work should evaluate the performance of the quality management systems that have been implemented in these companies.

Chapter 3 MICROBIOTA OF FROZEN *PANGASIUS* PRODUCTS MARKETED IN BELGIUM

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ABSTRACT

This chapter presents an overview of the microbiota of frozen Vietnamese *Pangasius* products marketed in Belgium. Samples of *Pangasius* steaks, portions and fillets from six brands were collected from supermarkets located in Ghent, Belgium. The total psychrotrophic and mesophilic aerobic counts of the samples evaluated from each brand did not differ significantly ($p > 0.05$) and ranged from 3.8-5.2 log CFU/g and 3.8-4.8 log CFU/g, respectively. Lactic acid bacteria counts varied from 2.2 to 4.1 log CFU/g while the counts of presumptive *Enterobacteriaceae* ranged from 1.6 to 3.8 log CFU/g. A total of 132 isolates were collected from the plates used to enumerate the microbial parameters mentioned above. Fourteen different genera and 18 different species were identified by means of 16S rRNA gene sequencing. The most prevalent genera were *Lactococcus* (31.2%), *Staphylococcus* (11.7%), *Serratia* (10.4%), *Acinetobacter* (9.1%), *Enterococcus* (7.8%) and *Pseudomonas* spp. (6.5%). The results obtained provide an overview of the microbiota of frozen *Pangasius* which is useful for the development of appropriate preservation techniques for thawed *Pangasius* products.

3.1 INTRODUCTION

In **Chapter 2** a high prevalence of *Aeromonas, Acinetobacter, Lactococcus* and *Enterococcus* spp. was found on *Pangasius* fillets during processing at two companies in Vietnam. It was also determined that the microbial diversity on the products depended on the location, source of water, suppliers (fish farms) and production capacity (**Chapter 2**). The dominant microbiota of different forms of *Pangasius* was recently reported i.e. on thawed *Pangasius* fillets stored in air and MAP conditions (Noseda et al., 2012) and on imported frozen Vietnamese *Pangasius* products retailed in Denmark (Noor Uddin et al., 2013). The microbiota on frozen products could influence the shelf life of thawed products, moreover, the trading of thawed products as (re)fresh fish is common in Western countries. Therefore, identification of the prevalent microbiota on frozen *Pangasius* products will provide an overview of the microbiota of frozen products which would allow processors to select appropriate preservation methods for thawed products.

The major objective of this chapter was to determine the microbiota of frozen Vietnamese *Pangasius* products sold in Belgium by means of a combination of culture-dependent techniques and 16S rRNA gene sequencing.

3.2 MATERIALS AND METHODS

Six different brands of frozen Vietnamese *Pangasius* products sold in various retail outlets in Ghent (Belgium) were evaluated. Four brands of the *Pangasius* products were in the form of fillets (*ca.* 200-220 g/fillet), one brand in the form of steaks (*ca.* 70-100 g/steak) and one other brand in the form of portions (portions & pieces are cut from frozen blocks *ca.* 70-80 g/piece). Three packages of each brand were purchased at the same time and kept at -20°C until the microbiological and chemical analyses were performed. Glazing of the fillet samples consituted 10% (brand 2 & 3 fillets), 6% (brand 4 fillets), 20% (brand 5 steaks) of the weight, whilst this was not known for brand 1 fillets and brand 6 portions. Addtional information regarding the composition (<1% citric acid and <1% salt) was provided on the labels of brand 3 fillets. Before the analyses were performed, the samples were initially thawed over a 24 h period in a refrigerator at 4.0 ± 0.7°C.

3.2.1 Drip loss, water content, water activity, pH and salt content

The drip (thawing) loss, water content, water activity (a_w), pH and salt content of all the *Pangasius* products were determined as follows. The drip loss was determined as the difference (%) between the weight of the packaged *Pangasius* products after thawing with and without the exudates. The weight of the packages was determined after thawing before the exudates were removed by decanting after which the weight of the package was measured again. Thereafter a 150-200 g composite sample from each package was homogenised for 1 min in a commercial blender (Braun 600W, Spain). The a_w and pH of the homogenates were measured in duplicate by means of a_w-kryometer (NAGY, Gaeufelden, Germany) and a SevenEasy pH meter (Mettler Toledo GmbH, Schwerzenbach, Swizerland), respectively. The water content of each sample was determined in duplicate gravimetrically by drying a 5 g aliquot of homogenate in aluminium dishes containing sea sand to avoid spattering for 12 h at 105°C. The salt was extracted by boiling a 5 g homogenate in distilled water for 10 min. The chloride content in the extract was determined by titration with silver nitrate (Merck, Darmstadt, Germany) using a 5% (w/v) chromate indicator (Merck, Darmstadt, Germany) according to the Mohr method (ISO 9297:1989).

3.2.2 Microbiological analyses

The fish samples for microbiological analyses were prepared separately with the fish samples of physico-chemical analyses above. A 150-200 g composite sample from each package was prepared for microbial analysis. The procedures of microbiological analyses were performed in this chapter as described in § **2.2.4** of **Chapter 2**. The total psychrotrophic and mesophilic aerobic counts were determined by pour plating the decimal dilutions on Plate Count Agar (PCA, Oxoid, Basingstoke, U.K.) followed by incubation for 72 h at 22°C and 30°C, respectively. The counts of presumptive *Enterobacteriaceae* were determined by pour plating (with an additional over layer) the decimal dilutions on Violet Red Bile Glucose agar (VRBGA, Oxoid, Basingstoke, U.K.). The VRBGA plates were incubated for 24 h at 37°C after which all colonies were counted. Psychrotrophic lactic acid bacteria (LAB) were determined by pour plating (with an additional over layer) the decimal dilutions on de Man Rogosa Sharpe agar (MRS, Oxoid, Basingstoke, U.K**)** followed by incubation for 72 h at 22°C.

3.2.3 Isolation and identification of dominant microbiota

From the three samples evaluated of each brand, 20-30 isolates were selected for identification taking into account as many different morphologies (e.g. color, size, and shape) as possible. These originated from the PCA, VRBGA, and MRS plates used for enumeration. A total of 132 isolates were purified by successive 4 × 4 streak plating (and microscopic analysis). The DNA extraction, rep-PCR and identification of the microbial isolates by sequence analysis was described previously in § **2.2.6.2 - 2.2.6.4** of **Chapter 2**.

3.2.4 Statistical analysis

Results of the physico-chemical characteristics and the microbiological analysis (log CFU/g) were reported as mean value ± standard deviation of triplicates for product (brand). Differences in the mean counts (log CFU/g) of the sampled products were statistically assessed using one way Analysis of Variance (ANOVA) in SPSS version 20 (IBM Inc., Chicago, Ill., USA) when a Shapiro-Wilk test indicated that the means were normally distributed. If a Levene test confirmed heteroscedasticity, a Tamhane's T2 test was used. A non-parametric Kruskal-Wallis H-type test was performed in case the data showed non-normality. Thereafter, comparison of the paired means was done using the Mann-Whitney U test ($\alpha = 0.05$).

3.3 RESULTS

3.3.1 Physico-chemical characteristics of frozen **Pangasius** *marketed in Belgium*

The results of physico-chemical characteristics performed on the samples are shown in **Table 3.1**. The mean water content of the thawed *Pangasius* fillets ranged from 79.3 to 87.7%. Fillets from brand 3 had significantly higher water content than the fillets from the other brands. The *Pangasius* steaks evaluated had significantly lower water content (74.0%) than the fillets and portions ($p < 0.05$). The mean drip (thaw) losses of the fillets ranged from 7.5 to 16.8%. The drip losses of fillets from brand 4 (mean = 7.5%) were significantly the lowest ($p < 0.05$) of the four brands of filleted *Pangasius* products evaluated. The portions had the smallest drip losses ($p < 0.05$) of any of the products evaluated; these being on average *ca*. 3 and 6 times lower than those fillets of brand 4 and 1, respectively. No correlation occurred between the water content and drip losses. The mean a_w values of the fillets ranged from 0.990 to 0.995, with fillets from brand 3 (a_w 0.990) having significantly lower a_w ($p < 0.05$)

than those of fillets from brand 1 and 2. The steaks and portions had a_w values (both a mean of 0.994) which were in the same range as fillets.

Table 3.1 Physico-chemical characteristics of Vietnamese *Pangasius* products marketed in Belgium

Product type	Water content (g/100 g wet fish)	Drip loss (%)	a_w	pH	Salt content (%)
Fillets (brand 1)	79.3 ± 1.2^{b}*	16.8 ± 0.2^{e}	0.9950 ± 0.0001^{c}	6.5 ± 0.0^{b}	0.12 ± 0.0^{a}
Fillets (brand 2)	80.5 ± 1.5^{b}	10.5 ± 1.7^{d}	0.9947 ± 0.0002^{c}	6.7 ± 0.3^{b}	0.28 ± 0.2^{abcd}
Fillets (brand 3)	87.7 ± 0.9^{c}	11.9 ± 3.9^{d}	0.9896 ± 0.0007^{a}	8.2 ± 0.2^{c}	0.93 ± 0.2^{d}
Fillets (brand 4)	80.0 ± 0.3^{b}	7.5 ± 0.1^{c}	0.9947 ± 0.0003^{abc}	6.5 ± 0.1^{b}	0.23 ± 0.1^{b}
Steaks (brand 5)	74.0 ± 2.1^{a}	12.6 ± 1.8^{bd}	0.9939 ± 0.0008^{b}	6.2 ± 0.1^{a}	0.49 ± 0.1^{c}
Portions (brand 6)	80.0 ± 1.4^{b}	2.6 ± 1.4^{a}	0.9944 ± 0.0002^{bc}	6.5 ± 0.1^{b}	0.22 ± 0.1^{ab}

*Data are expressed as mean value ± standard deviation of three replicates. Means with a different superscript letter in the same column indicate where statistically ($p \leq 0.05$) differences occurred between products.

The mean pH values of the fillets ranged from 6.5 to 8.2. The pH of the *Pangasius* fillets from brand 3 (mean = 8.2) were significantly higher ($p < 0.05$) than those of the fillets from the other three brands evaluated. The mean pH values of the portions did not differ significantly ($p > 0.05$) from that of fillets from brands 1, 2 and 4, whilst the *Pangasius* steaks (mean pH value = 6.2) had significantly lower pH values ($p < 0.05$) than those of the fillets and portions. The salt content (based on the chloride content) of the fillets ranged from 0.12 to 0.93%. As for the pH, the NaCl content of the *Pangasius* fillets from brand 3 (mean = 0.93%) were significantly higher ($p < 0.05$) than those of the fillets from the other three brands evaluated. The portions had a similar NaCl content to fillets of brands 1, 2 and 4 whilst the steaks had a mean NaCl content (0.49%) which was significantly higher ($p < 0.05$) than those of the portions and fillets of brands 1 and 4, but significantly smaller ($p < 0.05$) than that of fillets from brand 3.

3.3.2 Microbiota of frozen Pangasius *fish*

The microbial quality of frozen *Pangasius* products marketed in Belgium is shown in Error! Not a valid bookmark self-reference.. The total psychrotrophic aerobic counts (TPC) ranged from 3.8 to 5.2 log CFU/g, whilst the total mesophilic aerobic counts (TMC) ranged from 3.8 to 4.8 CFU/g. The TPC and TMC for each brand of fish did not differ significantly from each other ($p > 0.05$). With regards to the fillets, it can be seen that the TPC and TMC on fillets from brand 2 were both significantly lower ($p < 0.05$) than those on fillets from the other three brands. The TPC and TMC of the steaks and portions were significantly higher ($p < 0.05$) than those observed on fillets from brand 2. The counts of lactic acid bacteria (LAB) varied greatly between products, with significantly lower ($p < 0.05$) LAB occurring on the fillets from brand 2 than those found on the fillets from the other three brands. However, LAB counts from brand 2 did not differ significantly ($p > 0.05$) from these counts on the steaks and portions sampled. The counts of presumptive *Enterobacteriaceae* were highest on the fillets from brand 1 (3.8 ± 0.2 log CFU/g) while the lowest counts were found on the portions from brand 6 (1.6 ± 0.6 log CFU/g).

Table 3.2 Microbiota of Vietnamese *Pangasius* products marketed in Belgium

Product type	Total psychrotrophic aerobic counts (TPC)	Total mesophilic aerobic counts (TMC)	Lactic acid bacteria (LAB)	Presumptive *Enterobacteriaceae*
Fillets (brand 1)	$4.7 \pm 0.3^{cd*}$	4.8 ± 0.4^{c}	4.0 ± 0.7^{c}	3.8 ± 0.2^{d}
Fillets (brand 2)	3.8 ± 0.1^{a}	3.8 ± 0.0^{a}	2.2 ± 0.5^{ab}	2.9 ± 0.0^{c}
Fillets (brand 3)	5.2 ± 0.2^{c}	4.5 ± 0.3^{cb}	4.1 ± 0.1^{c}	3.0 ± 0.4^{cb}
Fillets (brand 4)	5.1 ± 0.4^{cd}	4.6 ± 0.2^{c}	4.0 ± 0.1^{c}	2.9 ± 0.2^{c}
Steaks (brand 5)	4.6 ± 0.2^{bd}	4.8 ± 0.1^{c}	2.7 ± 0.1^{b}	2.5 ± 0.2^{b}
Portions (brand 6)	4.4 ± 0.1^{b}	4.3 ± 0.1^{b}	2.3 ± 0.1^{a}	1.6 ± 0.6^{a}

*Data are expressed as mean value ± standard deviation (log CFU/g) of three replicates. Means with a different superscript letter in the same column indicate where statistically ($p \leq 0.05$) differences occurred between products.

3.3.3 Identification of the isolates collected from different products

A total of 132 isolates were collected from the plates used to enumerate the aerobic counts, presumptive *Enterobacteriaceae* and lactic acid bacteria on the frozen *Pangasius* products evaluated in this chapter. These isolates were clustered based on their rep-PCR fingerprints. Each cluster consisted of at least four isolates with a similarity level of at least 65%. From this cluster analysis, two representative isolates of each cluster were selected for further analysis by 16S rRNA gene sequencing and thereafter the tentative identification was extrapolated for the entire group of isolates in each cluster.

The 77 identified isolates (of which 36 isolates were Gram-negative and 41 were Gram-positive) included 14 different genera and 18 different species (**Table 3.3)**. On the basis of the total number of isolates identified, *Acinetobacter*, *Serratia*, *Staphylococcus* and *Lactococcus* spp. showed highly frequent, showing 9.1, 10.4, 11.7, and 31.2% of the isolates, respectively. *Lactococcus* spp. were isolated from five of the six brands evaluated, the only exception was fillets from brand 3. *Enterococcus*, *Stenotrophomonas*, *Chryseobacterium* and *Empedobacter* spp. were found only on fillets from brand 3. In addition, *Serratia* spp. were identified on the portions and steaks while *Enterobacter* and *Morganella* spp. were identified only on fillets from brand 1 and 4, respectively. *Staphylococcus* spp. was identified on the portions and fillets (from brands 1 and 2). The others microbiota were identified on *Pangasius* sampled including *Klebsiella* (brand 1 and 5), *Pseudomonas* (brand 1 and 4), *Arthrobacter* (brand 2), *Macrococcus* spp. (brand 1).

Table 3.3 Genera and species isolated from different *Pangasius* products sold in Belgium

Identification[1]	Fillets (brand 1)	Fillets (brand 2)	Fillets (brand 3)	Fillets (brand 4)	Steaks (brand 5)	Portions (brand 6)	Total isolates	Prevalence (%)
Acinetobacter	**1**[2]		**3**		**3**		7	9.1
Acinetobacter johnsonii					2			
Acinetobacter beijerinckii	1		3					
Acinetobacter haemolyticus					1			
Pseudomonas	**2**			**3**			5	6.5
Pseudomonas mosselii				3				
Pseudomonas beteli	2							
Stenotrophomonas			**1**				**1**	1.3
Stenotrophomonas maltophilia			1					
Serratia					**4**	**4**	**8**	10.4
Serratia nematodiphila					4	4		
Enterobacter	**4**						4	5.2
Enterobacter hormaechei	4							
Klebsiella	**1**				**2**		3	3.9
Klebsiella pneumoniae	1				2			
Morganella				**2**			2	2.6
Morganella morganii				2				
Chryseobacterium			**2**				2	2.6
Chryseobacterium indologenes			2					
Arthrobacter		**2**					2	2.6
Arthrobacter protophormiae		2						
Lactococcus	**1**	**3**		**8**	**7**	**5**	**24**	31.2
Lactococcus garvieae		3		8	7	5		
Lactococcus lactis	1							
Enterococcus			**6**				6	7.8
Enterococcus casseliflavus			6					
Macrococcus	**2**						2	2.6
Macrococcus caseolyticus	2							
Staphylococcus	**2**	**1**				**6**	**9**	11.7
Staphylococcus sciuri	2	1				6		
Empedobacter			**2**				2	2.6
Empedobacter brevis			2					
Total identified isolates	13	6	14	13	16	15	**77**	100
Species	7	3	5	3	5	3		

[1]Identification results on genus and species level; species identifications are only tentative

[2]The frequency of identified isolates based on rep-clustering and partial 16S rRNA gene sequence analysis with cut-off value of 98.5% similarity with type strains of validly published prokaryotic names in EZTaxon database. The percentage of total number of isolates of each genus is listed in the last column.

3.4 DISCUSSION

3.4.1 Physico-chemical characteristics

The results of the physico-chemical characteristics analyses of the frozen Vietnamese *Pangasius* products marketed in Belgium generally confirmed the findings of previous studies on *Pangasius* products. In agreement with our findings, Usydus et al. (2011) determined that the water content of frozen Vietnamese *Pangasius* products marketed in Poland was 84.7 ± 0.3%. Karl et al. (2010) reported that frozen Vietnamese *Pangasius* products marketed in Germany had water contents which ranged from 78.1 to 83.3%, drip losses between 12.5- 24.6% and pH values between 6.3-7.6. Orban et al. (2008) reported that frozen Vietnamese *Pangasius* products marketed in Italy had water contents which ranged from 80.1 to 85.0%, and pH values between 7.56-7.96. In the same study, a high sodium content (0.222-0.594%) was determined in the *Pangasius* products. This was assumed to be a result of the fish being possibly treated with water-binding additives of polyphosphates before freezing (Orban et al., 2008). The same conclusion was also derived by Karl et al. (2010) for *Pangasius* products marked in Germany by means of differential scanning calorimetry which showed a decreased thermal stability in the protein domains of the fish. The use of phosphates in both fish and meat can increase water retention and reduce thaw loss as a result of an increase in the pH and ionic strength and binding of phosphate to the protein (Gonçalves et al., 2008; Kaufmann et al., 2005; Thorarinsdottir et al., 2001). Brand 3 fillets were most likely treated with water-binding additives as they had the highest water content (87.7 ± 0.9%), lowest water activity (0.9896 ± 0.0007), highest pH (8.2 ± 0.2) and a very high salt content (0.93 ± 0.2%).

3.4.2 Microbiota of frozen **Pangasius** *products marketed in Belgium*

The TPC (3.8 to 5.2 log CFU/g) on the *Pangasius* products were not significantly different ($p > 0.05$) from the TMC (3.8 to 4.8 log CFU/g). This is consistent with previous results observed on frozen *Pangasius* originating from Vietnam that were processed for export to Belgium and other European countries (Noseda et al., 2012) & **Chapter 2**. The lactic acid bacteria (LAB) counts varied greatly between the products. The highest LAB counts, 4.1 ± 0.1 log CFU/g, were observed on the fillets from brand 3. These counts were in agreement with those obtained by Noseda et al. (2012), who found 3.9 ± 0.1 log CFU/g of LAB on thawed frozen Vietnamese *Pangasius* intended for a study regarding the effect of modified

atmosphere packaging. Of the LAB identified in the samples, *Lactococcus* spp. were the most prevalent (31.2%). These results were in agreement with the findings on *Pangasius* fish during processing (§ **2.3.3** in **Chapter 2)**. *Lactococcus* and *Enterococcus* spp. have previously been isolated from lightly preserved salmon products such as cold smoked, salted and dried (Leroi, 2010). *Lactococcus lactis* was found on traditional salted or dried Himalayan fish (Thapa et al., 2006) and *Lactococcus garvieae* was determined to be involved in the infectious diseases of fish (Vendrell et al., 2006). Both *L. lactis* and *L. garvieae* are also associated with fresh and marine water in tropical areas (Michel et al., 2007). In addition to their association with fish farm environments, they are also sometimes isolated from human and other mammalian clinical cases (Michel et al., 2007).

With regards to the presumptive *Enterobacteriaceae, Enterobacter, Klebsiella, Morganella* and *Serratia* spp. were identified. The incidence of these isolates appeared to be dependent on how the frozen *Pangasius* was processed as *Serratia* was isolated from steaks and portioned *Pangasius* products, whilst *Enterobacter* and *Klebsiella* were isolated from fillets (brand 1) and *Morganella* from fillets (brand 4). Differences between the types of *Enterobacteriaceae* contaminating frozen *Pangasius* products in Vietnam have also been found on the basis of the size of the processing plant. Frozen *Pangasius* fillets processed in a large plant were determined to be contaminated by *Serratia* spp. whereas those processed in a small scale plant were contaminated by *Enterobacter, Klebsiella,* and *Morganella* spp. (§ **2.3.3** in **Chapter 2**). Kim et al. (2003) also pointed to the importance of sanitation in the fish processing plant to prevent cross-contamination from *Enterobacter, Klebsiella,* and *Morganella*. Moreover, species belonging to the *Enterobacteriaceae* family are in general frequently isolated from tropical and farmed fish (ICMSF, 2005) and Vietnamese *Pangasius* (Sarter et al., 2007). It has been suggested that *Enterobacter, Klebsiella, Morganella* and *Serratia* spp. may be good representative species of the microbial ecology of *Pangasius* fish. Furthermore, the counts of presumptive *Enterobacteriaceae* were highly variable among products, ranging from 1.6 ± 0.6 to 3.8 ± 0.2 log CFU/g. It has to be mentioned that the enumeration approach used in this chapter can present an idea of the presumptive *Enterobacteriaceae* as *Acinetobacter* and *Pseudomonas* spp. were also identified from isolates growing not only on non-selective PCA but also on selective VRBGA plates.

The ability of *Acinetobacter* spp. to grow on both non-selective (PCA) and selective media (VRBGA), may have contributed to the high prevalence of *Acinetobacter* spp. (9.1%) on the

frozen *Pangasius* fish evaluated. In addition, *Acinetobacter* and *Pseudomonas* spp. have been isolated from the intestines of fish (Hovda et al., 2007; Merrifield et al., 2009; Ringø et al., 2006) and therefore may potentially contaminate the fish during processing. This is supported by the previous findings where *Acinetobacter* and *Pseudomonas* spp. were detected on *Pangasius* samples collected at the filleting and trimming steps during processing in Vietnamese companies (§ **2.3.3** in **Chapter 2**)**.** Moreover, *Pseudomonas* spp. have also been determined to be the dominant spoilage bacteria on thawed *Pangasius* stored in air at 4°C (Noseda et al., 2012). The spoilage capacity of the isolates identified here should be further evaluated to provide better insights into their spoilage mechanisms.

The prevalence of *Staphylococcus sciuri* (11.7%) on frozen *Pangasius* was relatively high. *Staphylococcus* spp. are very common in humans and therefore they could have been transferred to the products *via* human contact during handling and processing. *S. sciuri* is the most frequently reported histamine-forming bacterium in cod, escolar steaks, swordfish fillets, cold smoked rainbow trout and whole and filleted catfish (Chang et al., 2008; Hwang et al., 2012; Ramos and Lyon, 2000).

Empedobacter, *Macrococcus*, *Arthrobacter*, *Chryseobacterium* and *Stenotrophomonas* spp. were less prevalent in the frozen *Pangasius* products evaluated. *Stenotrophomonas maltophilia,* an important opportunistic pathogen, has been also isolated from channel catfish in China (Geng et al., 2010). *Chryseobacterium* spp. has also been found on *Pangasius* fish sampled during processing in Vietnam (§ **2.3.3** in **Chapter 2**) and frozen *Pangasius* exported to Denmark (Noor Uddin et al., 2013). *Chryseobacterium indologenes* has been isolated from diseased yellow perch (Pridgeon et al., 2013) whilst *Chryseobacterium* spp. are known to be widely distributed in the environment and soil (Benmalek et al., 2010), and fresh water (Kim et al., 2008; Park et al., 2008).

3.5 CONCLUSION

A high prevalence of *Pseudomonas* (6.5%), *Enterococcus* (7.8%), *Acinetobacter* (9.1%), *Serratia* (10.4%), *Staphylococcus* (11.7%) and *Lactococcus* spp. (31.2%) was determined on thawed Vietnamese *Pangasius* products marketed in Belgium. These results are crucial as currently very little is known about the microbiota of thawed *Pangasius* products marketed in the West as 'fresh' *Pangasius* products. This knowledge is important with regards to the development of suitable preservation techniques such as vacuum and modified atmosphere packaging to inhibit the microorganisms contaminating thawed *Pangasius* fish.

Chapter 4 DYNAMICS OF MICROBIOLOGICAL SAFETY AND QUALITY OF *PANGASIUS* FILLETS IN A LARGE AND SMALL SCALE VIETNAMESE PROCESSING COMPANY

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Tong Thi, A.N, Jacxsens, L., Noseda, B., Samapundo, S., Nguyen, B., Heyndrickx, M., and Devlieghere, F., 2014. Evaluation of the microbiological safety and quality of Vietnamese *Pangasius hypophthalmus* during processing by a microbial assessment scheme in combination with a self-assessment questionnaire. *Fisheries Science*,1-12.

ABSTRACT

Vietnamese Tra fish (*Pangasius hypophthalmus)* have become highly appreciated by consumers in the European Union, USA, Canada, etc. and are therefore of worldwide economic importance. However, the availability of data on the microbiological quality and safety of this fish species is limited. The dynamics of microbiological performance between large and small scale Vietnamese *Pangasius* processing plants (where non-chlorinated water and chlorinated water was used during the washing steps, respectively) were evaluated from the raw materials until final product by means of a microbial assessment scheme (MAS). A total of 279 samples (135 samples from the large scale plant) were taken to assess the overall microbial quality (psychrotrophic aerobic count), hygiene indicators (*Escherichia coli* and *Staphylococcus aureus*), and relevant pathogens (*Listeria monocytogenes* and *Vibrio cholerae*). Low levels of total psychrotrophic bacteria (*ca.* 3 log CFU/g) and *E. coli* (below quantification limit) were found on the final products sampled from the large scale plant. In addition, *Listeria monocytogenes* and *Vibrio cholerae* were absent in all the samples analysed. On the contrary, high numbers of psychrotrophic bacteria (*ca.* 6 log CFU/g on fish and *ca.* 6 log CFU/100 cm^2 on food contact surfaces) were found in the small scale plant during processing. Additionally, foodborne pathogens were present in the water, hands and fish; especially *L. monocytogenes* as a result of inadequate hygiene practices in the processing environment. Also discussed in this chapter are the results of a self-assessment questionnaire, which provide insight into the performance of the food safety management system currently implemented at these companies.

4.1 INTRODUCTION

Vietnam has over the last few years been the largest exporter of *Pangasius* products to the U.S. Vietnamese *Pangasius* were ranked the sixth favorite fish species in the U.S. in 2011 (FAO, 2012). Although Vietnamese *Pangasius* products are accepted as being of good nutritional quality and safety (Karl et al., 2010), the control of (cross) contamination by pathogens is still challenging. Pathogenic bacteria can be transmitted into aquaculture products during rearing, handling and processing as a result of improper hygienic conditions. For example, *Salmonella* spp., *Vibrio cholerae* and *Listeria monocytogenes* originate from rearing ponds or the processing environment (Reilly and Kaeferstein, 1998). Moreover, between 2005 and 2013, the RASFF reported cases of rejection of Vietnamese *Pangasius* products destined for European countries due to the presence of pathogens such as *Salmonella* spp. and *Listeria monocytogenes* (**Table 1.6** of **Chapter 1**). In **Chapter 2**, it is emphasized that in addition to very high counts of presumptive *Enterobacteriaceae,* several pathogenic species of *Enterobacteriaceae* (i.e. *Shigella flexneri*) also occurred on *Pangasius* products from small scale processing plants in Vietnam. However, there is still very little data on the transmission routes of human pathogenic bacteria during the handling of *Pangasius* products and the microbiological quality and safety of final products.

Despite the large economic value of *Pangasius* products to Vietnam, little research has been conducted on the performance of the food safety management systems (FSMS) implemented at *Pangasius* processing companies and their influence on the microbiological quality and safety during processing. Previously, some studies emphasized that different food processing plants can deal with different microbial loads and food safety issues due to variability in implementing and understanding of the performance of FSMS (Cormier et al., 2007). To know if the FSMS is performing adequately, the number of microorganisms and variation of microbial counts could be assessed throughout the processing chains by means of a microbial assessment scheme (MAS) (Jacxsens et al., 2009b). The assessment scheme is a vertically microbiological sampling plan throughout the production process, from raw materials to final products. Such a microbiological sampling plan has previously been applied to gain insight in the production processes of various types of foods (§ **1.4** in **Chapter 1**). To our knowledge, no study has yet been performed on *Pangasius* fish.

In this study, the microbiological quality and safety of *Pangasius* was determined during processing in two Vietnamese companies. These were a large scale plant where the washing steps were done with non-chlorinated water and a small scale plant where chlorinated water was used during the

washing steps. Both plants are substantially oriented to export of frozen *Pangasius* fillets. The FSMS currently implemented in processing Vietnamese Tra fish was evaluated by means of a MAS throughout the entire production process. In addition, assessment of the context, control and assurance activities, and food safety output of the FSMS applied was performed by a self-assessment questionnaire (Jacxsens et al., 2011; Luning et al., 2008; Luning et al., 2009b)

4.2 MATERIALS AND METHODS

4.2.1 Microbial Assessment Scheme (MAS)

4.2.1.1 Characterization of the sampled company

A large scale factory with a potable water–based process and a small scale factory with a chlorinated water–based process previously visited to analyse the microbial ecology of *Pangasius* (**Chapter 2**) were evaluated in the present study to gather knowledge on the microbial safety and quality. The processing plant and the product manufacturing have been described in § **2.2.1** in **Chapter 2**.

4.2.1.2 Critical sampling locations (SL)

Critical sampling locations are locations in the production process at which contamination, growth and/or survival of microorganisms may occur due to loss of control at these locations. The samples collected in this chapter consisted of both fish fillets and environmental samples i.e. water and food contact surfaces, hand or glove swabs. The SLs included the raw material at the beginning of the process, production processes like trimming, filleting, water baths used for washing and packaging, the food contact surfaces like knives and work tables, hands and/or gloves of the operators and the final packaged product. The positions of the SLs in the flow of the production process for this study are indicated in **Figure 4.1 A** (big) and **B** (small company).

4.2.1.3 Sampling frequency

These companies were visited three times over a 4-week period and three different times (*ca.* 8 a.m., 12 a.m. and 2 p.m.) during each visit. The samples were collected at 15 SLs (total of 135 samples: 54 *Pangasius* samples, 27 swabs of hands/gloves, 27 swabs of food contact surfaces and 27 water samples) in the large scale plants (**Table 4.1**). For the small scale plants, the samples were collected at 16 SLs (total of 144 samples: 54 *Pangasius* samples, 36 swabs of hands/gloves, 27 swabs of food contact surfaces and 27 water samples) (**Table 4.2)**. However, the experiment was performed in February–March 2011 in the large scale plant and in March 2013 in the small scale plant.

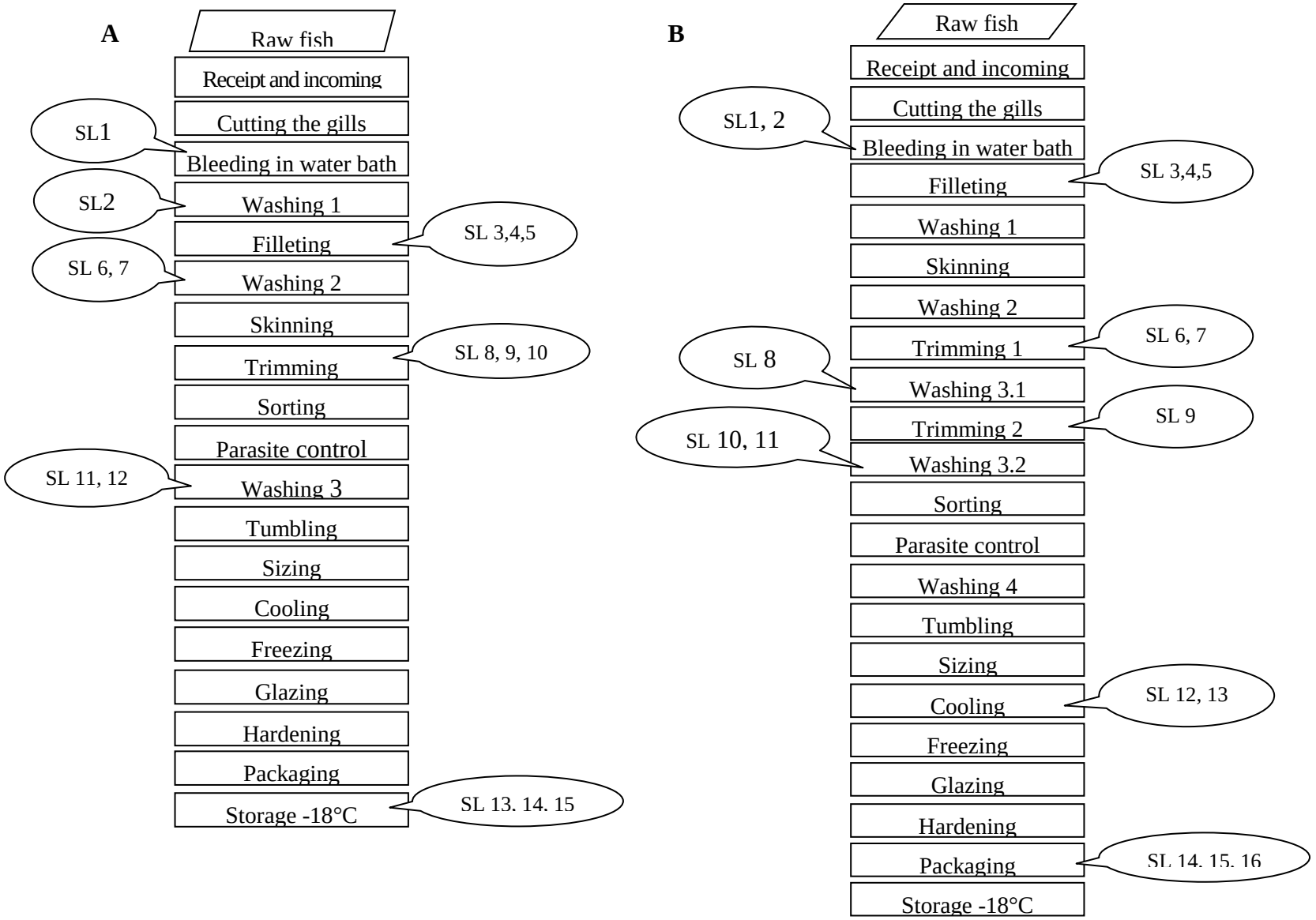


Figure 4.1: Flowchart of production process of *Pangasius* processing in the large company (A) and small company (B) indicating critical sampling locations (SL)

4.2.1.4 Sampling and analysis method

4.2.1.4.1 Sampling in large scale plant

Samples of the *Pangasius* fillets, hands/gloves, food contact surfaces and water were collected in duplicate. One sample was analysed immediately in the microbiological laboratory of the company in Dong Thap city, Vietnam (**Table 4.1)** and the other half of the samples were supplemented with 2% glycerol and stored frozen at -18°C. The samples were transported frozen to the LFMFP (Ghent, Belgium) where they were further stored at -21°C until analysis was performed. Analysis of the frozen samples occurred within one month of the sampling.

The overall microbial quality (i.e. total aerobic psychrotrophic counts and total aerobic mesophilic counts); hygiene indicators (*E. coli*, *Enterobacteriaceae, Staphylococcus aureus*, lactic acid bacteria), and pathogens (*Listeria monocytogenes*, *Vibrio cholerae*) were determined depending on the type of samples (**Table 4.1**).

Table 4.1 Overview of the microbiological parameters investigated at each sampling location (large scale plants), * samples analysed in Vietnam

Samples	Number	Sampling locations	Investigated parameters
Water	1 6 11	Bleeding Washing 2 Washing 3	Total aerobic mesophilic count (TMC)* Total aerobic psychrotrophic count (TPC) *E. coli* * *L. monocytogenes*
Fish	2 5 7 10 12 15	Bleeding Filleting Washing 2 Trimming Washing 3 Packaging	TMC* TPC Lactic acid bacteria (LAB) *Enterobacteriaceae* *E. coli* * *Staphylococcus aureus* *L. monocytogenes* *Vibrio* spp*; V. cholerae*
Hands or gloves	3 8 13	Filleting Trimming Packaging	TPC *E. coli* * *L. monocytogenes*
Food contact surfaces	4 9 14	Filleting Trimming Packaging	TPC *E. coli* * *L. monocytogenes*

4.2.1.4.2 Sampling in small scale plant

The small scale plant was located in Can Tho city. The same types of samples were collected in the small scale plant as those collected at the large scale plant. All samples were taken aseptically, stored in ice and transported in insulated boxes to the Laboratory of Microbiology and Biotechnology (Department of Food Technology, Can Tho University, Vietnam) for microbiological analyses within 24 h of sampling.

The overall microbial quality i.e. total aerobic psychrotrophic counts, hygiene indicators (*E. coli* and coliforms), personal hygiene indicators (*Staphylococcus aureus*) and pathogens (*Listeria monocytogenes*, *Salmonella* spp. and *Vibrio cholerae*) were determined depending on the type of sample (**Table 4.2**).

Table 4.2 Overview of the microbiological parameters investigated at each sampling location (small scale plants)

Samples	**Number**	**Sampling locations**	**Investigated parameters**
Water	1 8 10	Bleeding Washing 3.1 Washing 3.2	Total aerobic psychrotrophic count (TPC) *E. coli* Coliforms *Listeria monocytogenes* *Salmonella* *Vibrio cholerae*
Fish	2 5 9 11 13 16	Bleeding Filleting Trimming Washing 3.2 Cooling Packaging	TPC *E. coli* Coliforms *S. aureus* *Listeria monocytogenes* *Salmonella* *Vibrio cholerae*
Hands or gloves	3 6 12 14	Filleting Trimming Cooling Packaging	TPC *E. coli* Coliforms *S. aureus* *Listeria monocytogenes* *Salmonella* *Vibrio cholerae*
Food contact surfaces	4 7 15	Filleting Trimming Packaging	TPC *E. coli* Coliforms *Listeria monocytogenes* *Salmonella*

4.2.1.4.3 Microbiological analysis

For the fish samples, a *Pangasius* fillet (*ca.* 200 g) was aseptically taken with sterile tweezers and placed in stomacher bag. For food contact surfaces and the hands or gloves, swabs were taken vertically, horizontally and diagonally on a 100 cm^2 surface. In total, four swabs were taken. Before swabbing, sterile swabs (Copan, Italy) were pre-moistened in 5 ml Maximum Recovery Diluent (MRD, Merck, Darmstadt, Germany) for enumeration of total aerobic counts, LAB, *Enterobacteriaceae*, *E. coli*, coliforms and *Staphylococcus aureus* depending on sampled company (**Table 4.1** & **Table 4.2**); in 5 ml Demi-Fraser broth (Merck, Darmstadt, Germany) for detection of *L. monocytogenes*, in 5 ml buffered peptone water (Oxoid, Basingstoke, UK) for detection of *Salmonella* spp., and in 5 ml Alkaline Saline Peptone (pH = 8.6) (Merck, Darmstadt, Germany) for detection of *V. cholerae*. Every moistened swab was applied to each food contact surface, and then inserted back into its tube containing 5 ml of solution. With regards to the water samples, *ca*. 500 ml of water from three different locations in the water baths was collected in sterile stomacher bags. Thereafter, 1 ml of water was aseptically taken for microbiological analyses.

Quantitative microbiological analysis

For fish samples, 25 g of sample was aseptically taken from different parts of the fillet by means of sterile scalpels and tweezers and placed in a sterile stomacher bag. 225 ml of sterile MRD was then added after which the mixture was homogenized for 1 min in a stomacher. For water samples, 1 ml of water was aseptically transferred to 9 ml of MRD. The water samples (and also the swab samples in MRD) were vortexed for 10 s. Thereafter; a tenfold serial dilution series was performed. The total psychrotrophic and total mesophilic counts were determined on Aerobic Count Plate petrifilms™ (3M™ Microbiology Products, St. Paul, MN, USA) following incubation at 22°C for 72 h and 30°C for 72 h, respectively. Coliforms and *E. coli* were enumerated on Coliform/*E. coli* petrifilms™ (3M™ Microbiology Products, St. Paul, MN, USA) after incubation at 37°C for 24 h and 48 h, respectively (for samples in the small plant) whereas enumeration of *E. coli* was done on *E. coli* petrifilms™ (3M™ Microbiology Products, St. Paul, MN, USA) by incubating at 42°C for 24 h (for samples in the large plant). *S. aureus* was enumerated on Staph Express Count petrifilm™ (3M™ Microbiology Products, St. Paul, MN, USA) following by incubating at 35°C for 24 h (for samples in the small plant). *S. aureus* was enumerated by spread plating (1 ml/4 plates)

on Baird Parker (Oxoid, Hampshire, United Kingdom) agar plates with 25 ml/500 ml Egg Yolk Tellurite Emulsion (Oxoid, Hampshire, United Kingdom) after an incubation period of 48 h at 37°C and confirmation of *S. aureus* occurred with a coagulase test Staphytect Plus (Oxoid, Hampshire, United Kingdom) (for samples in the large plant). Psychrotrophic lactic acid bacteria were enumerated on Man Rogosa Sharpe agar (Oxoid, Hampshire, United Kingdom) by pour plating and anaerobic incubation of 72 h at 22°C. The number of *Enterobacteriaceae* was determined on Violet Red Bile Glucose agar (Oxoid, Hampshire, United Kingdom) pour plates, which were incubated 24 h at 37°C with a cover layer.

Qualitative analysis

Presence of L. monocytogenes

For the fish samples, 25 g of sample was added to 225 ml of Demi-Fraser broth. For the water samples, 1 ml of water sample was transferred to 4 ml of Demi-Fraser broth. The fish, water and swab samples (in Demi-Fraser broth) were then pre-enriched by incubation for 24 h at 30°C. Subsequently, 0.1 ml was inoculated in 10 ml of Fraser broth solution (Merck, Darmstadt, Germany) and incubated for 48 h at 37°C. This culture was then streaked on ALOA (Agar Listeria Ottaviani Agosti, Biolife, Milan, Italy) and incubated at 37°C for 48 h. Typical colonies of *L. monocytogenes* are a green-blue color surrounded by an opaque halo.

Presence of V. cholerae

Pre-enrichment of the fish (25 g of fish in 225 ml alkaline saline peptone water), water (1 ml of water samples in 4 ml of alkaline saline peptone water) and swab samples (in alkaline saline peptone water) was done by incubating for 6 h at 41.5°C, with the exception of the frozen samples which were incubated at 37°C. Subsequently, 1 ml of the pre-enriched sample cultures were inoculated into 10 ml of alkaline saline peptone water and incubated for 18 h at 41.5°C. A loopful of the second culture was then streaked onto the surface of thiosulfate citrate bile salts sucrose (TCBS) agar plates (Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. Thereafter, typical colonies (yellow and smooth colonies) were inoculated on Tryptone Soya Agar (TSA, Oxoid, Hampshire, United Kingdom) supplemented with 1.5% of NaCl (Merck, Darmstadt, Germany) for 24 h at 37°C for confirmation. Confirmation was on the basis that *V. cholerae* is Gram- negative and oxidase positive.

Presence of Salmonella spp.

Pre-enrichment of the fish (25 g of fish in 225 ml of buffered peptone water), water (1 ml of water samples in 4 ml of buffered peptone water) and swab samples (in buffered peptone water) was performed by incubation at 37°C for 18 h. Following pre-enrichment, 0.1 ml of the first pre-enrichment culture was transferred to 10 ml of Rappaport Vassilliadis Soya peptone broth (RVS, Oxoid, Basingstoke, UK). The inoculated RVS tubes were then incubated for 24 h at 41.5°C. A loopful of culture from the RVS tubes was streaked onto Xylose Lysine Deoxychlate agar (XLD, Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. Thereafter, typical *Salmonella* colonies were picked from the XLD plates and transferred to XLD slants. They were transported in this form to the Laboratory of Food Microbiology and Food Preservation (Ghent University, Belgium) for further phenotypical and serological confirmation tests.

4.2.1.5 Data processing and interpretation of results

Interpretation of the results of the fish and food contact surfaces was based on the legal criteria established by the Vietnamese Ministry of Science and Technology (TCVN, 2010) and the microbial criteria (guidelines) recommended by the Laboratory of Food Microbiology and Food Preservation (LFMFP, Ghent University, Belgium) (Uyttendaele et al., 2010) (see **Table 4.3**). The initial quality of water used for washing fish must meet potable water standards according to EU Council Directive 98/83/EC (EU, 1998b) and Vietnamese regulation (TCVN, 2009). However, no guidelines or criteria were available for the microbial quality of the water when it is reused to wash several batches of fish. However, the self-checking guide mentions that the washing process may not lead to further contamination of the products, and the frequency of refilling/refreshing water in the baths should be determined by company.

After the evaluation of each parameter for each SL, a **microbiological safety level profile** was defined according to the method of Jacxsens et al. (2009b). The microbiological safety level is scored 1 to 3. Level 3 reflects a good Food Safety (FS) performance, meaning that legal criteria and microbiological guidelines are respected and thus that the current FSMS is covering the hazards in an adequate way. Level 2 indicates a moderate FS performance. Level 1 reveals a poor FS performance. Legal criteria and microbiological guidelines are

exceeded and improvements of the current FSMS are needed on multiple activities of the FSMS.

Table 4.3 Legal requirements or guideline values for microbiological parameters for microbial assessment

Microbial parameters	Fresh fish in Belgian food industry[a] (log CFU/g)		Frozen tra fish fillets[b] (log CFU/g)	Food contact surfaces[a] (log CFU/100 cm^2)
	Goal	Tolerance	Tolerance	
Total aerobic counts	5	6	6	Good, ≤ 3; moderate 3-4.5; poor ≥ 4.5
Enterobacteriaceae //Coliforms[*]	2	3	x	Good, ≤ 3; moderate 3-4.5; poor ≥ 4.5
Psychrotrophic lactic acid bacteria (LAB)	2	3	x	x
E. coli	2	3	2	Absence in area tested
Staphylococcus aureus	2	3	2	Absence in area tested
V. cholerae	x	x	Absence in 25g	Absence in area tested
L. monocytogenes	Absence in 25g	Absence in 25g	x	Absence in area tested
Salmonella	Absence in 25g	Absence in 25g	Absence in 25g	Absence in area tested

[a]According to guideline value for fresh fish developed by the Laboratory of Food Microbiology and Food Preservation (Ghent University) (Uyttendaele et al., 2010)

[b]According to microbiological criteria for production frozen Tra fish (*Pangasius hypophthalmus*) fillet established by Vietnamese Science & Technology Ministry (TCVN, 2010)

[*]No guidance value or criteria for coliforms; thus the guidance value of *Enterobacteriaceae* can be used for coliforms though coliforms belong to *Enterobacteriaceae* family

× not mentioned in the guideline for fresh fish or the criteria for frozen *Pangasius* fish

The sum of the microbiological safety levels for each parameter might reach a maximum of 27 of 9 (microbiological parameters) x 3 (levels) for the large (or 7 x 3 = 21 for the small scale plant). When the microbiological profile is lower than 27 for the large (or 21 for the small scale plant), there is room for improvement of the current FSMS to mitigate microbiological hazards or improve the quality.

Based on the microbiological safety profile, a score was assigned to express the overall FS performance of the current FSMS (Jacxsens et al., 2009b; Sampers et al., 2010).

The assigned score is 1 if sum of microbiological safety levels ranges 9-11 (or 7-8)
The assigned score is 1_2 if sum of microbiological safety levels ranges 12-16 (or 9-12),
The assigned score is 2 if sum of microbiological safety levels ranges 17-21 (or 13-15),
The assigned score is 2_3 if sum of microbiological safety levels ranges 22-25 (or 16-19),
The assigned score is 3 if sum of microbiological safety levels ranges 26-27 (or 20-21).

4.2.1.6 Statistical analysis

The results of the microbiological analysis of the water, fish and swab samples were expressed as log CFU/ml, log CFU/g and log CFU/100 cm^2, respectively. The results are reported in this chapter as the mean value ± standard deviation. Differences in the mean microbial counts between processing steps or during the three different visits and three independent sampling times were statistically assessed using a non-parametric Kruskal-Wallis H type test in SPSS version 20 version (IBM Inc., Chicago, Ill., USA) due to the data showing non-normally and comparison between paired means was performed using the Mann-Whitney U test. A non-parametric Spearman rank order correlation coefficient (r) was calculated for cross-correlations between the microbiological counts investigated with a two tailed test ($\alpha = 0.05$).

4.2.2 Self-assessment questionnaire on food safety management system (FSMS-DI)

A questionnaire of FSMS-DI (a diagnostic self-assessment) with 58 indicators was designed based on the work Luning et al. (2008), Luning et al. (2009b) and Jacxsens et al. (2011). The questions were categorized under the following topics: (a) context factors (i.e. product characteristics, production process, organization, and chain environment), (b) control activities (i.e. preventive measures, intervention processes, monitoring system design and their operation), (c) assurance activities (i.e. setting system requirements, validation,

verification activities, documentation and record-keeping) and (d) the food safety performance. The questionnaire was answered by the people responsible for quality assurance (QA) at the company *via* in-depth interviews took *ca.* 3 h to conduct.

Of the 58 questions, 17 assessed the context factors, 25 assessed the control activities, nine assessed the assurance (these indicators described in **Table 1.7**) and seven assessed the food safety performance. The seventeen questions on the context were graded as situation 1, 2, or 3 which corresponded to low, potential, or high vulnerability (to safety problems), ambiguity (lack of insight in underlying mechanisms), and uncertainty (lack of information), respectively (Luning et al., 2009b). For the twenty five questions on the control activities, four levels were defined 0, 1, 2 or 3, which corresponded to not relevant, incomplete, guidelines-based or science-based/fit-for-purpose, respectively. The nine assurance activities questions also comprised four levels: 0, 1, 2 and 3 referring to unknown, historical knowledge (but no analysis), restricted and comprehensive levels, respectively (Luning et al., 2008). All seven questions about the food safety performance were defined into four levels: 0, 1, 2 and 3, referring to absent/not measured, minimum follow-up, standard follow-up and comprehensive system evaluation, respectively (Jacxsens et al., 2011).

For each question, the interviewees had to select which situation or activity level was the most representative for their company. Each question was well defined and designed by "if then" combined with supportive information to guide the interviewees in advance during interview.

The food safety management system diagnosis resulted in a list of scores for the separate questions for contextual factors, for control and assurance activities which were summarized into spiderweb diagrams. Then, a mean value was calculated based on the sum of the scores for the separate questions divided by the total number of questions. Mean scores were transformed to an assigned level/situation score as follows:

- If mean situation score of major *contextual factor* is between 1-1.2 then assigned situation 1,
- If between 1.3-1.7, assigned score 1-2,
- If between 1.8-2.2, assigned score 2,
- If between 2.3-2.7, assigned score 2-3 and
- If between 2.8-3.0, assigned score 3.

Similarly,

- If mean score for *core control/assurance activity* is between 0-0.2, then assigned score 0.
- If between 0.3-1.2, assigned score 1,
- If between 1.3-1.7, assigned score 1-2,
- If between 1.8-2.2, assigned score 2,
- If between 2.3-2.7, assigned score 2-3 and
- If between 2.8-3.0, assigned score 3.

The assigned scores can be used to obtain an overall indication (impression). However, to search for possible points for improvement, one needs to look to the individual scores as well (Luning et al., 2011a; Luning et al., 2011b).

4.3 RESULTS

4.3.1 Microbial quality and safety of Pangasius *fillets processed in the companies*

A total of 279 samples (135 samples in large scale plant) were analysed to establish the microbiological quality of *Pangasius* fillets, water and food contact surfaces during processing. Distribution of the total aerobic psychrotrophic counts (TPC) of samples of fish, environment and water samples during the three visits or the three sampling times are shown in **Figure 4.2 A&B** where it can be seen that higher TPC counts occurred on the samples collected at the small scale plant**.** There was no significant difference in the TPC counts of fish, water, hands and food contact surfaces during the three visits to the large scale plant ($p > 0.05$) whilst the food contact surfaces and water samples were significantly different ($p < 0.05$) in this plant. The fish and hands samples were not significantly different ($p > 0.05$) during the visits to the small scale plant. A comparison was also made between the three different times of sampling; the only significant difference was observed for the fish samples whose counts increased significantly in the samples collected during the second sampling ($p < 0.05$). No significant differences ($p > 0.05$) were observed in the TPCs of the hands, food contact surfaces and water samples collected during the three sampling times in the large scale plant. Unlike the large scale plant, no significant differences ($p > 0.05$) occurred in TPC counts of fish samples collected during the three sampling times at the small scale plant, whilst in similairy to the large scale plant no significant differences ($p > 0.05$) occurred in

TPC counts water, hands and food contact surfaces during the three sampling times in the small scale plant (**Figure 4.2**).

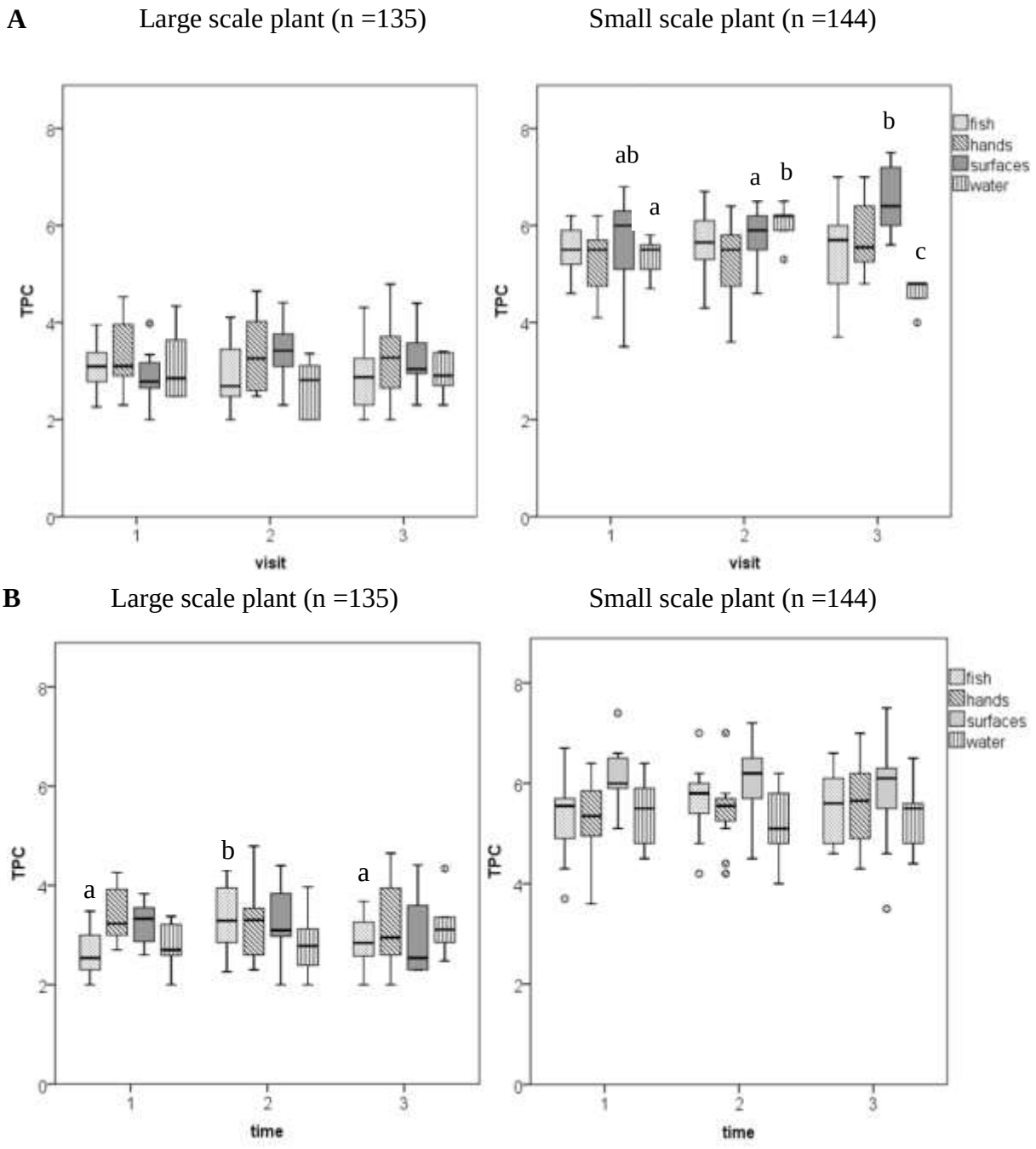


Figure 4.2 The distribution of total psychrotrophic counts (TPC) between visits (A) and between sampling times (B). X-axis by visit: 1 (*ca.* 8a.m), 2 (*ca.* 12a.m) and 3 (*ca.* 14 p.m). X-axis by time: day 1, day 2 and day 3. Y-axis: log CFU/g (fish), log CFU/100 cm^2 (hands and surfaces) and log CFU/ml (water). A value with a diferent letter on samples between visits or times in the same company sampled shows statistical significance ($p < 0.05$).

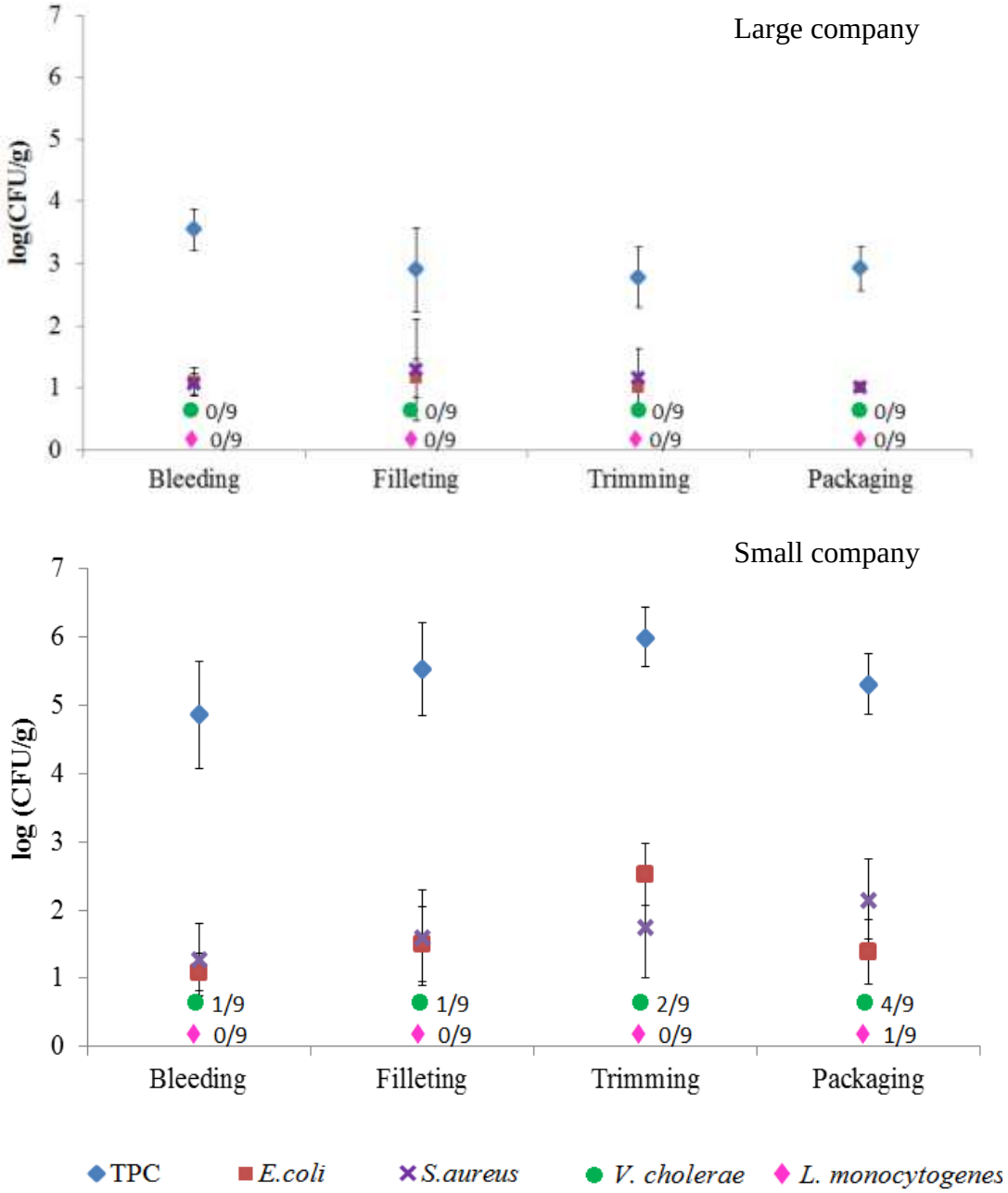


Figure 4.3 The microbiological profiles of fish samples between the large and small scale plants

L. monocytogenes and *V. chlolerae* were not detected **in the large company** (**Figure 4.3 and Figure 4.4).** The results of the microbiological quality of the *Pangasius* fish at different processing locations can be seen in **Figure 4.3.** TPC counts reduced during processing from 3.6 ± 0.3 (raw materials) to 2.9 ± 0.4 log CFU/g (frozen products) (**Figure 4.3**). TPC on the food contact surfaces were 2.9 ± 1.8, 2.7 ± 0.5 and 1.4 ± 1.7 log CFU/100 cm^2 at the filleting, trimming and packaging steps, respectively. TPC on hands were significantly reduced during processing steps, to be 4.0 ± 0.7, 3.0 ± 0.7 and 2.3 ± 1.3 log CFU/100 cm^2 at the filleting, trimming and packaging steps, respectively (**Figure 4.4)**. *E. coli* was only found sporadically

and in low numbers, ranging from 1.0 to 1.2 log CFU/g on fillets (**Figure 4.3)** and at counts lower than the quantification limit (<1 log CFU/100 cm^2) on the hands/gloves and surfaces samples (**Figure 4.4**). Lactic acid bacteria (LAB) were present in relatively high numbers (i.e. 3.0 log CFU/g of fish at the filleting step), six (out of 54) samples exceeded the goal levels (2 log CFU/g). For *Enterobacteriaceae,* only one fillet sample had counts which exceeded the goal level (2 log CFU/g). For TMC, there were two samples exceeding the goal value (5 log CFU/g).

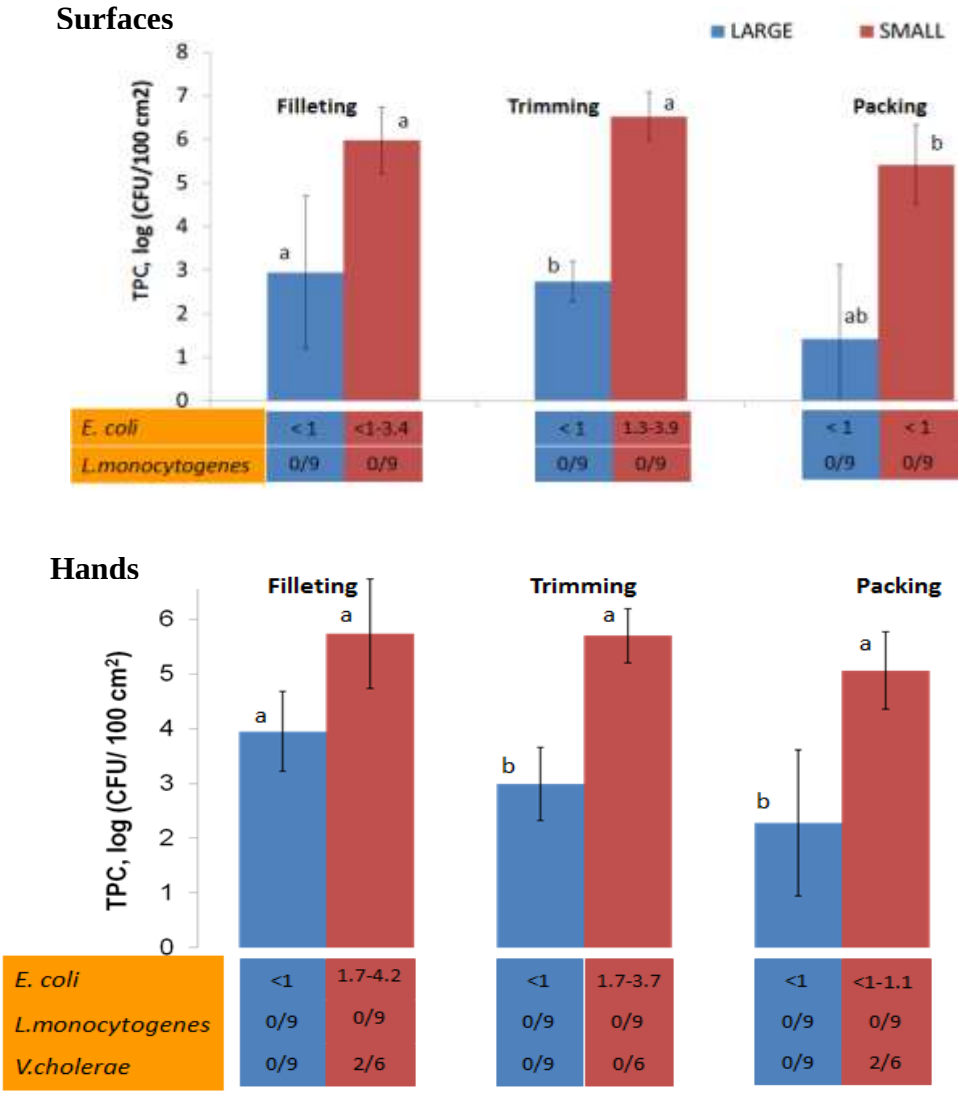


Figure 4.4 The microbiological counts on hands and surfaces of the large and small scale plants. Value with a different letter between processing steps in the same company sampled shows statistical significance ($p < 0.05$).

The results of the microbiological quality of the *Pangasius* fish at different processing locations **in the small company** can be seen in **Figure 4.3.** In general, the microbial counts (except for

Salmonella which was absent) during processing exceeded the goal level. It is seen in the **Figure 4.3** that TPC increased from 4.9 ± 0.8 log CFU/g on the raw materials to 6.0 ± 0.4 log CFU/g on the fillets sampled at the trimming step. Then, TPC decreased slightly until they were 5.3 ± 0.4 log CFU/g on the fillet samples collected after packaging, the final processing step. Evolution of *E. coli* counts during processing followed the trend observed for TPC. *S. aureus* occurred at low levels on samples of the raw material (1.4 ± 0.5 log CFU/g), followed by increased gradually up to 2.3 ± 0.6 log CFU/g on the final products. *L. monocytogenes* was isolated from only one sample of the final product (a frozen *Pangasius* fillet), whilst *V. cholerae* was sporadically isolated from *Pangasius* samples at different processing steps: bleeding (1/9 samples), filleting (1/9), trimming (2/9) and packaging (4/9) (**Figure 4.3**). The pathogenic bacteria of *Salmonella* spp. were absent in all fish samples investigated.

The TPCs on the food contact surfaces ranged from 5.4-6 log CFU/100 cm^2 while those on the hands/gloves ranged from 5.1-5.7 log CFU/100 cm^2 at the filleting, trimming and packaging steps. On the hands/gloves, *E. coli* was found at the highest levels at the filleting step, ranging from 1.7-4.2 log CFU/100 cm^2 while the highest contamination of *E. coli* on the food contact surfaces (tables, knives etc.) was found at the trimming step (1.3-3.4 log CFU/100 cm^2). *S. aureus* counts on the hands/gloves ranged <1-1.9 log CFU/100 cm^2 at the filleting, trimming and packaging steps, respectively. *V. cholerae* was also detected on the hands/gloves of personnel at the filleting (2/6 samples), cooling (2/6) and packaging (2/6) steps. *L. monocytogenes* was not detected on the hands/gloves and food contact surfaces (**Figure 4.4**).

The aerobic psychrotrophic counts in the water samples ranged from 4.4-5.9, 4.0-6.4 and 4.5-6.5 log CFU/ ml at the bleeding step, and at washing steps 3.1 and 3.2, respectively. *E. coli* was detected at low numbers in the water samples. As an example the *E. coli* counts ranged from below the limit of quantification (<1 log CFU/ml) to 1.7 log CFU/ml in water from the bleeding bath. *L. monocytogenes* and *Salmonella* spp. were absent in all the water samples collected, whilst only one sample of water collected from washing step 3.1 was contaminated with *V. cholerae.*

In addition, a strong correlation was observed between the counts of *E. coli* and coliforms (r^2 = 0.747, p = 0.000, n = 144). The correlation among the other microbiological parameters was not strong as *E. coli* and coliforms. More specifically, correlation coefficients of 0.434, 0.522 and 0.211 were obtained between TPC and *E. coli* counts (p = 0.000, n = 144), coliforms (p = 0.000, n = 144), and *S. aureus* (p = 0.045, n = 90), respectively.

Based on the MAS results, the **food safety** level was calculated to be 20 (/27) in the large scale and 9 (/21) in the small scale plant. The overall MAS score was assigned at 2 and 1_2 in the large and small scale plant, respectively (**Table 4.4**).

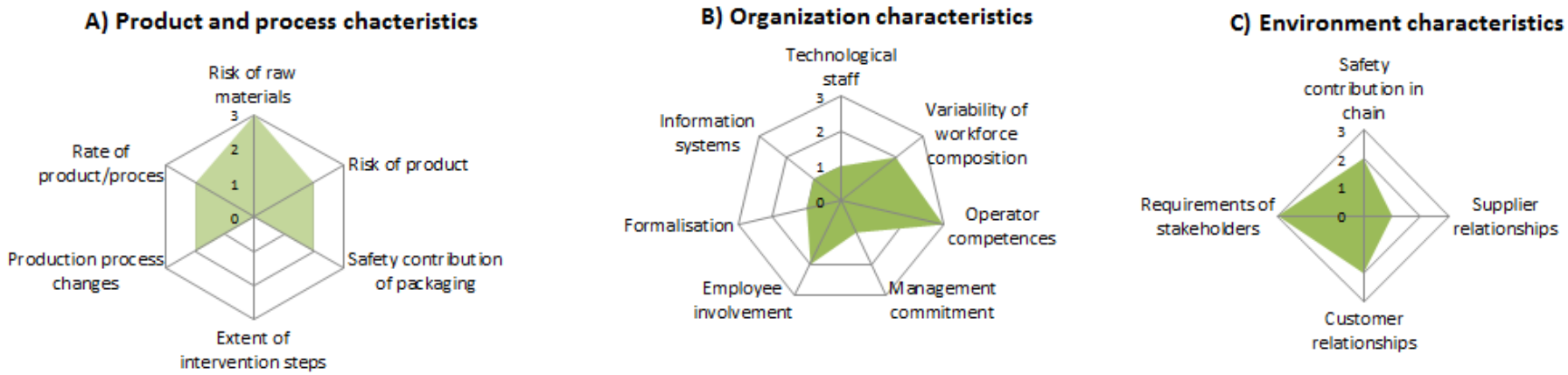
4.3.2 Results of self-assessment questionnaire

Table 4.4 Assigned score for the contextual factors, food safety control & assurance (FSMS), food safety performance indicators (FSPI) and microbiological safety level (FS) for large and small scale plant.

Assigned score	Contextual factor	FSMS	FSPI	FS
Large company	2	2_3	2_3	2
Small company	2_3	1_2	1_2	1_2

The basic assumption behind the FSMS-DI (a diagnostic self-assessment) is that companies working with riskier products and processes (context 3) need a more advanced FSMS (level 3) to be able to comply with safety requirements than companies operating in a less risky contextual situation (context level 2 or 1). The context factors (**Table 4.4**) show that both companies evaluated in this study were assigned a score of 2 or 3. The detailed scores of the contextual factor showed in **Figure 4.5.** The FSMS is separated into two major parts: food safety control activities and food safety assurance activities. Indicators for the food safety core control activities comprise technology-dependent and managerial activities in design and operation of preventive measures, intervention processes, monitoring systems and operation control measures (**Figure 4.6**). The food safety core assurance activities are dealing with validation, verification, documentation and record keeping and defining a system set-up**.** Both the food safety control activities and the food safety assurance activities at the large scale company had an overall assigned score of 2_3 **(Table 4.4).** A clear difference was seen in the assigned scores of 1_2 for the control and assurance activities at the small scale company (**Table 4.4**). Analysis of individual scores of core control activities are shown in **Figure 4.6** and **Figure 4.7** for the large and small scale company, respectively. In general, the assurance activities of the small scale company were on level 1 (historical knowledge) with 44% (4 out of 9) of the responses and level 2 (restricted level) with 56 % (5 out of 9) of the responses. In contrast to the results in the large scale company, these activities were elaborated at higher levels (i.e. 33.3% of the responses at level 3 and 55.6% at level 2). The scores of the food safety performance indicators (FSPI) were 1_2 and 2_3 for the small and large scale company, respectively (**Table 4.4**).

Large scale plant



Small scale plant

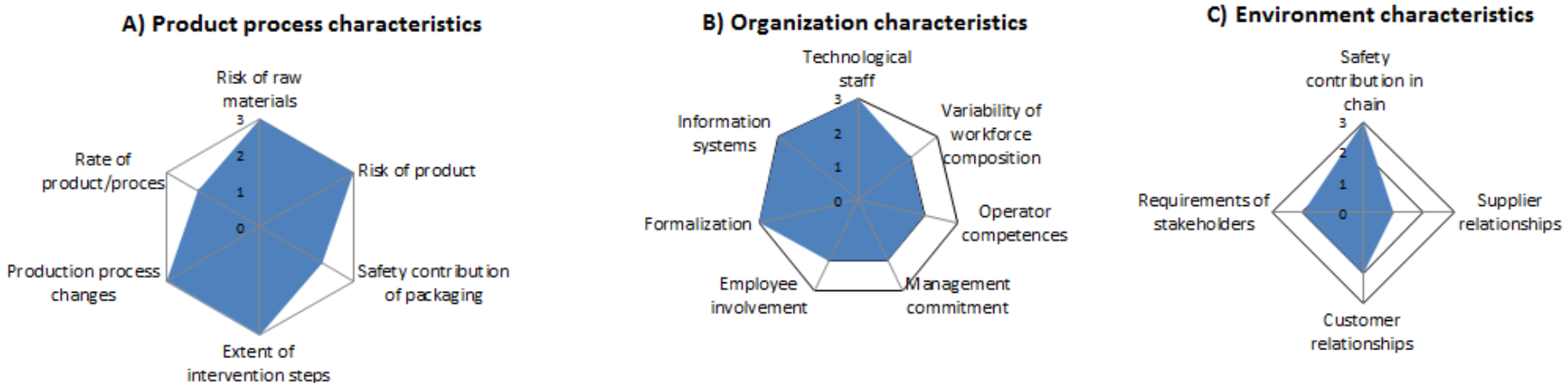


Figure 4.5 Spider webs of the scores awarded for the contextual factors of large and small company

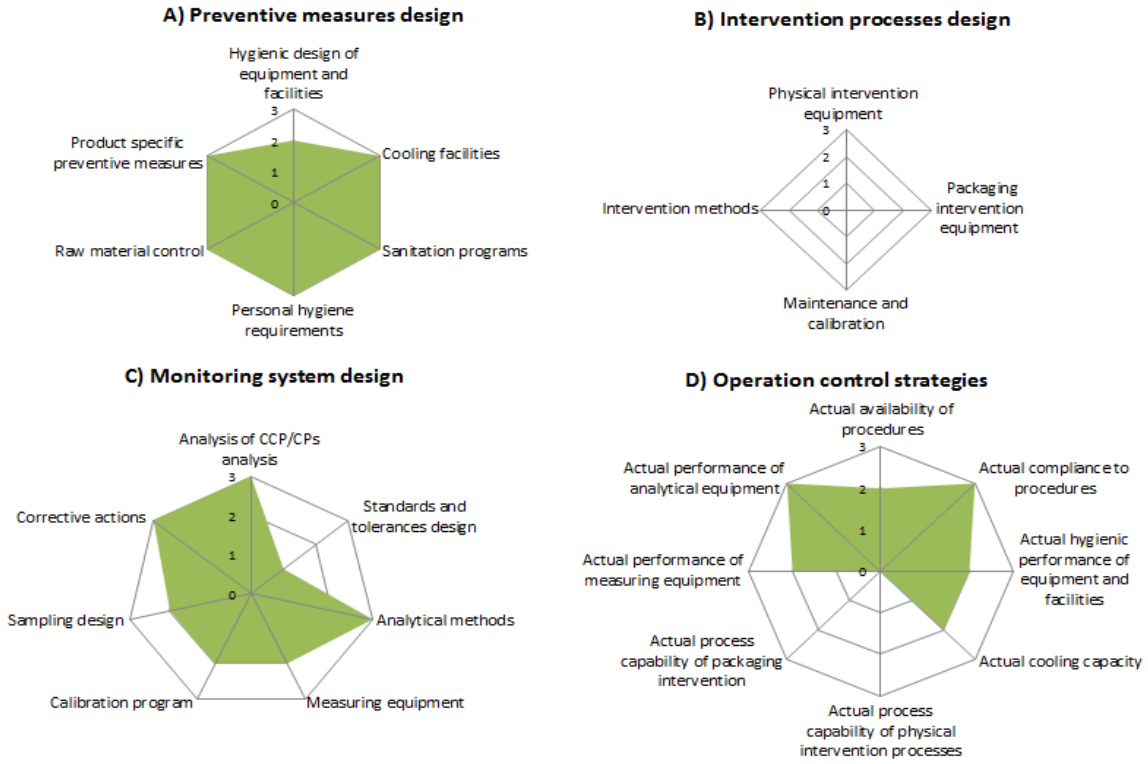


Figure 4.6 Spider webs of the scores awarded for the core control activities of the large scale plant

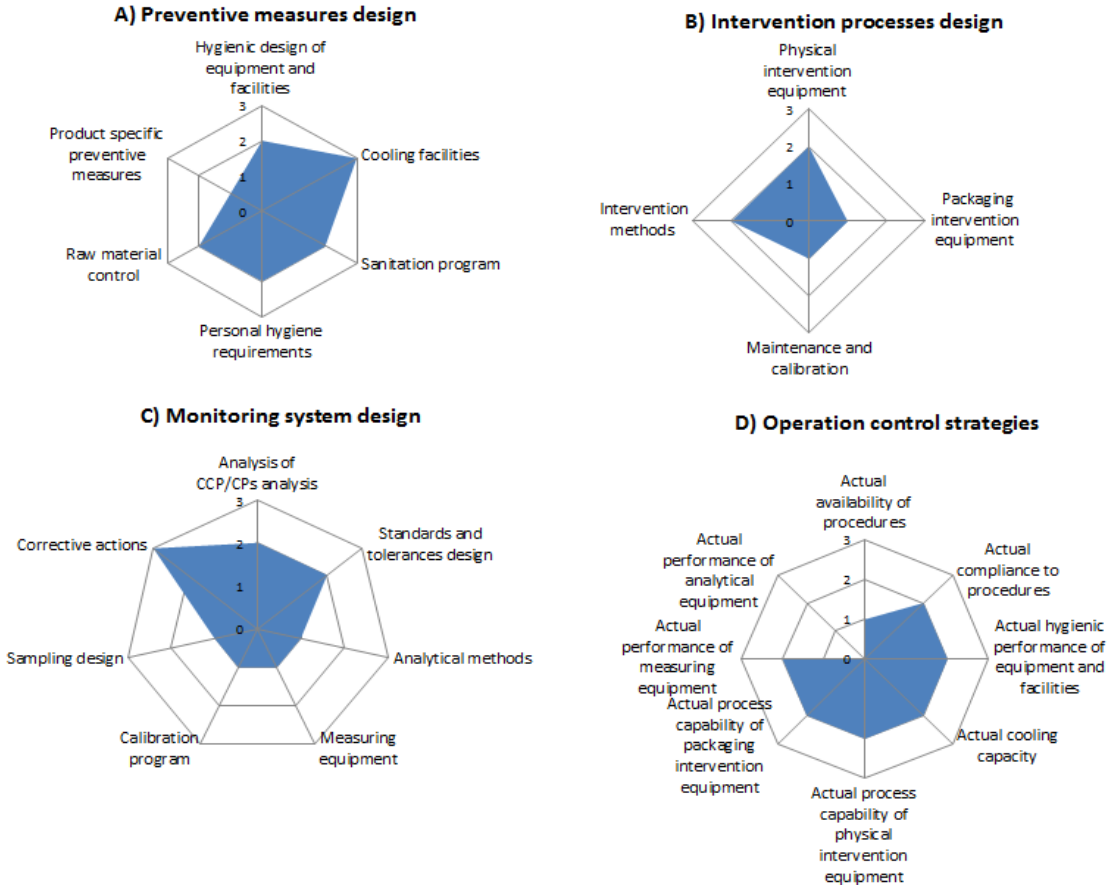
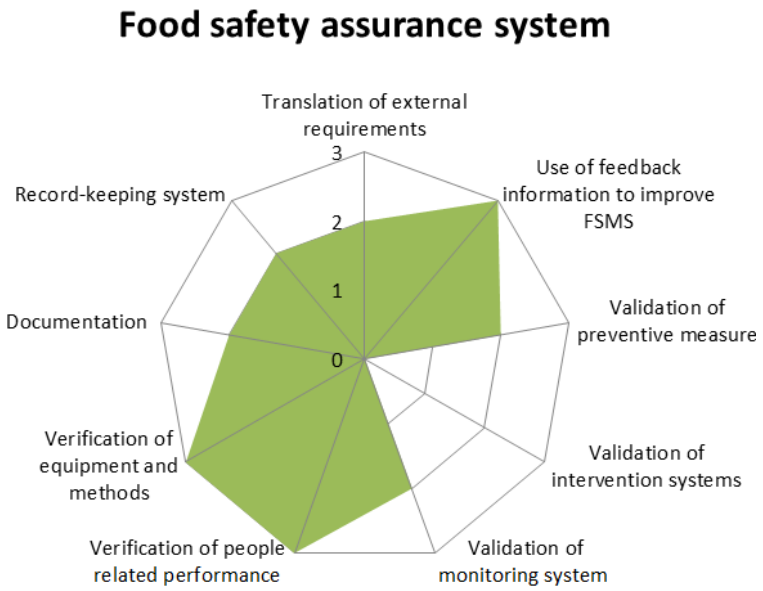


Figure 4.7 Spider webs of the scores awarded for the core control activities of the small scale plant

Large scale plant



Small scale plant

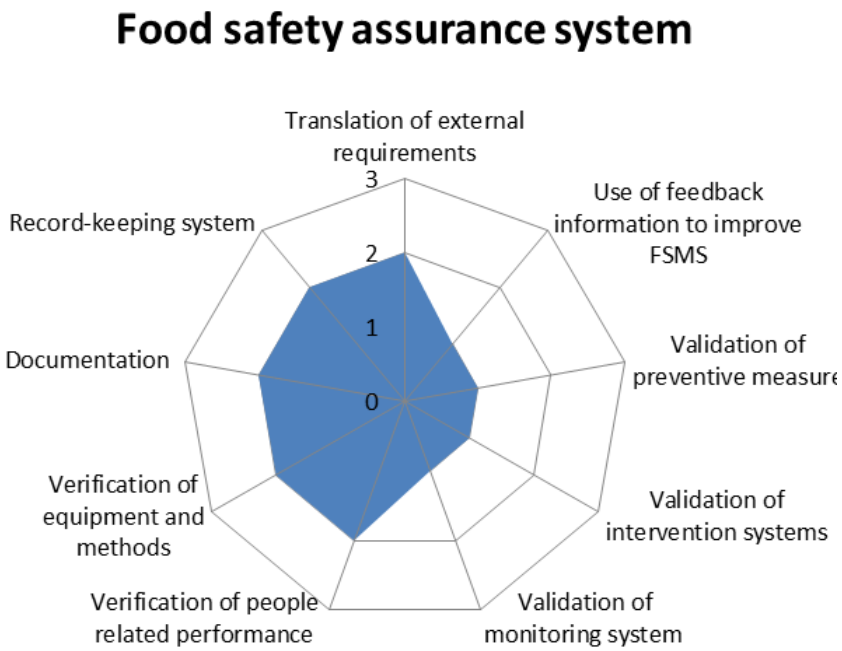


Figure 4.8 Spider webs of the scores awarded for the core assurance activities of the large and small scale plant

4.4 DISCUSSION

4.4.1 Microbial quality and safety of **Pangasius** *fillets during processing evaluated by means of microbiological assessment scheme*

The MAS results provided insight into the actual microbiological safety and quality of *Pangasius* fish processed in both different scale plants in Vietnam. The assumption made in this study was that the FSMS of the companies are performing at advanced levels, meaning that the microbiological counts on *Pangasius* fish during processing should be lower than the tolerance limits according to the guidelines for fish products after production (TCVN, 2010; Uyttendaele et al., 2010) (**Table 4.3**). In the small scale plant, the total aerobic psychrotrophic counts (TPC) of the final products (4.6-5.9 log CFU/g) exceeded the goal limit of 5 log CFU/g. In addition, these counts were greater than those on the products sampled from a large scale company (2.5-3.6 log CFU/g). These counts were in a good agreement with those observed in **Chapter 2**, where the microbial ecology during processing was evaluated in the same companies.

The hygiene indicators, consisting of *E. coli* and coliform counts, varied widely. As an example, counts of *E. coli* observed in the fish sampled from the small scale plant varied between <1-3.5 log CFU/g whilst sporadic and low counts of *E. coli* were observed in the fish (<1-1.3 log CFU/g) sampled in the large scale plant. In this study, the coliforms counts were *ca*. 1 log CFU/g higher than those of *E. coli* and a significant positive correlation (r = 0.747) occurred between these two microbial parameters. The correlation between these two microbial parameters supports the idea that coliform counts could be used to predict the counts of *E. coli* (Hood et al., 1983). However, Leclercq et al. (2002) recommended the replacement of coliforms analyses by *E. coli* enumeration as a means of estimating the sanitary quality of food. In addition, *E. coli* enumeration would likely give useful information as a quality indicator of fishery products, particularly the quality of *Pangasius* products (TCVN, 2010; Uyttendaele et al., 2010).

Most coliforms are present in large numbers in diverse natural environments, the intestinal microbiota of humans and other warm-blooded animals, and are therefore harbored in fecal waste or freshwater bottom sediments or sands (Pachepsky and Shelton, 2011). *E. coli* is the most common coliform in the intestinal microbiota of warm-blooded animals and is thought to be principally associated with fecal contamination (Rompré et al., 2002). A previous study

has reported that *Enterobacteriaceae* (including *E. coli*) originate from the intestines of tropical freshwater fish (Apun et al., 1999) although their incidence was sporadic and low counts were found in the large scale plant. It should be taken into consideration that the enumeration of *Enterobacteriaceae* occurred after frozen transport to Belgium, so the original contamination during production might have been higher as freezing can reduce their numbers (Reinartz et al., 2011). The origin of *E. coli* and *Enterobacteriaceae* is also likely to be related to the *Pangasius* gut. Low numbers of *Enterobacteriaceae* (2.5 log CFU/g) and of *E. coli* (1.5 log CFU/g) were recovered from the gut of a frozen *Pangasius* sample (data not shown). Additionally, higher counts of *Enterobacteriaceae* (4.2 ± 0.1 log CFU/g) were found in the gut of a *Pangasius* sample (§ **2.4.1** in **Chapter 2)**. Repetitive observations of the filleting step revealed that perforations of the gut by the knives used during this manipulation occur between 28% and 55% of the times depending on the operator. This is a possible transmission route and explains the sporadic presence of *E. coli* and *Enterobacteriaceae* on the fillets. On the other hand, cross contamination can occur when bacteria are transferred from food contact surfaces (i.e. hands, cutting boards and knives) to the food. High counts of both coliforms and *E. coli* were found on the hands and surface samples collected during processing of the small scale plant, indicating insufficient hygiene practices in the small scale company investigated in this study.

In the small scale company, high levels of contamination were found in the water used to wash the fish. The fillets were washed manually by shaking a basket filled with 10 kg of fillets in tap water (*ca.* 100 litres) in washing step 3.1 to remove dirt, fat and red muscle from the surface of the fillets. High TPC (4.0-6.4), *E. coli* (<1-2.6) and coliform (2.3-3.6 log CFU/ml) counts and the presence of *V. cholerae* (on 1 of 9 samples) were found in the water used at washing step 3.1. Therefore, there is a risk of cross contamination with pathogens from the washing water to the fish fillets. Moreover, to improve the microbial quality of fish after the trimming step, the fish fillets were washed in washing step 3.2 in water with 50 ppm chlorine. Unexpectedly, TPC on *Pangasius* fillets before (6.1 ± 0.6 log CFU/g) (data not shown) and after washing (6.0 ± 0.4 log CFU/g) were equal. Therefore, the fillets might not be decontaminated during washing. In addition, chlorinated water still showed high levels of bacteria e.g. 4.5-6.5, <1-2.6 and 2.3-3.5 log CFU/ml TPC, *E. coli* and coliforms, respectively. During the visit, 50 ppm NaOCl was prepared for use at washing step 3.2 just before the shift started without adjustment of the pH and the chlorine concentration during processing. This result suggests that the concentration of chlorine and the bacterial load in the washing water

as a function of time should be further evaluated to increase the efficacy of the process at the small company. In the large scale company, washing was done in a water bath containing only potable water and flake ice combined with an inflow of compressed air. The water was used for a single batch of washing (§ **2.2.2** in **Chapter 2)**. As a result, low microbial counts and no pathogens were detected in the samples of the washing water. Also no relevant reductions of the total mesophilic counts on the fillets were observed in this system. The major function of these water baths are likely cleaning the fillets (removal of blood, fat, etc.) and cooling the fillets before freezing.

V. cholerae was found in the water at washing step 3.1 of the small company. *V. cholerae* was also found at other sampling locations including the water at the bleeding step (1/9), on the hands of the workers at the filleting step (2/6) and on the *Pangasius* fish sampled at the filleting step (1/9). *V. cholerae,* is a natural inhabitant of aquatic environments and has been isolated from the digestive tracts of fish (Senderovich et al., 2010). This may explain why *V. cholerae* was detected on the fillets and the hands of the workers at the filleting step. In addition, *Vibrio* spp. were also found in tropical water environments and fish are actually considered as reservoirs of *V. cholerae* (Codex Alimentarius Commission, 2003). *V. cholerae* was also found on 4 out of 9 final packaged products from the small company. It might be a result of inadequate personnel hygiene at the small company. However, in contrast to the small company, *V. cholerae* was not found in *Pangasius* fillets samples from the large scale company.

In addition, *S. aureus,* as an indicator of hand hygiene, was found on the hands of food operators (up to 1.9 log CFU/100 cm^2) at the packaging step in the small company. The *S. aureus* counts on five of the nine samples evaluated were greater than the limit of quantification (1 log CFU/100 cm^2), indicating that the hygiene practices were inadequate (Uyttendaele et al., 2010). Moreover, *L. monocytogenes* was found on one of the nine samples of the final product. *L. monocytogenes* is commonly found in water where fish are captured or cultivated, and in contaminated freshwater fish (Yucel and Balci, 2010). The transmission of *L. monocytogenes* into the final product has been reported to occur from the fish raw materials and the processing environment (Chen et al., 2010; Miettinen and Wirtanen, 2006). Hansen et al. (2006) have reported that the incidence of *L. monocytogenes* is low in fish farms and the environment inside fish processing plants. The processing

environment has been reported as a route of transmission for *L. monocytogenes* into processed fish rather than directly from raw fish (Chen et al., 2010; Hoffman et al., 2003).

As a result of the presence of *L. monocytogenes* and *V. cholerae* in the final *Pangasius* products of the small scale company, it can be concluded that the high microbial quality does not conform to the microbiological guidelines nor to the criteria for frozen *Pangasius* fillets (TCVN, 2010; Uyttendaele et al., 2010). The hygiene levels and sanitation procedures in the production area should be revised as cleaning and sanitation programs can have a great impact on reducing the presence of *L. monocytogenes* in the factory environment (Hoffman et al., 2003; Huss et al., 2000). Taking into consideration the microbiological parameters discussed above, a microbial safety level profile of 1_2 (= poor food safety performance) was assigned to the small company compared to 2 (= moderate level) for the large company (**Table 4.4**).

4.4.2 Current performance of the food safety management system by means of self-assessment questionnaire

The assessment of contextual factors (**Figure 4.5**) indicated that these companies were operating in a moderate to high vulnerability context. The context situation, the product and process characteristics of the small scale company were similar to those observed in the large scale plant. Previous studies have also noted that the characteristics of the production process of *Pangasius* fillets in various companies in Vietnam are very similar (Karl et al., 2010). In contrast, the organizational characteristics such as formalization and technological staff were highly vulnerable (situation 3) in the small company whilst those of the large scale company were of low vulnerability (situation 1). The small company employs five people who are working in the quality assurance (QA) department and all microbial analyses or safety controls are performed by external laboratories whereas the large company has a significant quality assurance department with quite a large staff (> 15 persons) including experts in various aspects of food safety and has its own well equipped research lab. Another weak point of the small company was not only the absence of activities in formal procedures but also the lack of formalized meetings e.g. meetings of the quality department. At both companies evaluated, variability occurred in the workforce with a turnover of between 1 to 5 years. When experienced persons leave the company, knowledge is lost which may have an impact on food safety and food quality output. Since the small company does not have any

requirements regarding a basic education level or experience of the recruited operators and does not provide an intensive training program, the 'operator competences' are at level 2 in the small company, which poses a relative high risk for food safety and quality (Seaman, 2010). In addition, the information systems wherein information about safety processing, product, hazards is systematically recorded were less (not readily) accessible to the staff in the small company. The characteristic of the information systems seems dependent on the size of the company as the large company has a specific well accessible quality information system (**Figure 4.5B**). In the chain environment characteristics (**Figure 4.5C**), both companies have a very high influence on their suppliers of raw materials. The companies own half of their *Pangasius* supplying farms, meaning that the company can have a large bearing on the desired safety and quality of the raw materials. The 'customer relationships' are not able to put specific requirements on the quality system of the customers, which are mainly export destinations. The large company has to meet additional quality assurance requirements from their customers, which are different for the major stakeholders, e.g. the legal requirements that resulted in 'requirements of the stakeholders' (score of 3). Yet, conflicting requirements from stakeholders are also putting pressure on the production process and the FSMS, e.g. the application of chlorine as disinfection agent applied in the washing water, which is not allowed for European production (EU, 2004b) but is desired to reduce the bacterial load on products destined for export to the US. The mean score for all contextual factors was therefore assigned 2 and 2_3 in the large and small scale plant, respectively **(Figure 4.5** and **Table 4.4)**.

The FSMS had an assigned score of 2_3 **in the large company (Table 4.4)**, which is at a higher level than required by the contextual factors (score 2). The fact that the control and assurance activities were almost equal to each other means that the FSMS is well balanced in the large company. The control activities usually have a high score in most of the food processing companies that are in compliance with the food safety legislation and requirements (Luning et al., 2011b; Sampers et al., 2010). The assurance activities are important as the control activities guarantee a high food safety level. A high score for the assurance activities means that a company is really involved in providing a high food safety level of their products and is adequately documenting and validating their control activities (Luning et al., 2011b; Sampers et al., 2010).

The control activities in the large company are adequate (**Figure 4.6)**. In practice, the large company, for instance, has a systematic control of incoming raw materials based on the statistical analysis of actual historical data of the suppliers. The 'analysis of the CCP/CPs' (Critical Control Points/ Control Points) is executed in a systematic way and tested for the actual production circumstances (**Figure 4.6C)**. The company's laboratory is equipped for their own challenges test to monitor evolutions of inoculated pathogens on the product during simulated storage. For 'standards and tolerances design', the standards for critical product (e.g. water content) and process (e.g. pH tumblers) parameters are specified, but no tolerances are yet clearly specified. It would be advisable to improve this indicator by e.g. taking tolerance values from general hygiene codes (e.g. Code of Practice for fish and fishery products, CAC/RCP 52-2003, http://www.codexalimentarius.net), legal or stakeholder requirements and clearly specifying them. As the laboratory of the company can perform all conventional culture-based methods (mainly plate counts and absence-presence tests) and is accredited for these methods according to ISO 17025 (requirements for testing and calibration laboratories), the 'analytical methods to assess pathogen levels' is of an advanced level. Moreover, the lab is examining on regular basis *Salmonella, L. monocytogenes, E. coli*. Furthermore, samples are also double checked by sending them to external accredited labs. The 'corrective actions' were based on systematic causal analysis of the company's own product/process deviations. However, the 'measuring equipment to monitor the process/product status', the 'calibration program for measuring and analytical equipment' and the 'sampling design and measuring plan' are not specifically designed for the company's production process and therefore could be improved by validating them specifically to the company's production process in order to raise a desired level.

The core assurance activities are activities providing confidence that the desired safety requirements will be met. They deal with setting requirements on the system, evaluating its performance and organizing necessary changes (Luning et al., 2008). The core assurance activities in the large company were achieved at advanced (55.6% at level 2) to the most advanced level (33.3% at level 3). It indicates that the FSMS of the large company has a well-documented system where personnel procedures, equipment and methods are frequently verified by independent experts based on analyzing records (e.g. control charts, temperature data loggers, etc.), calibration activities and actual microbiological testing. The 'validation of intervention systems' was not taken into account in the final evaluation of this company because packaging is considered a mechanical barrier. The control activities of **the small**

company sampled was less advanced which means that they are designed or conducted based on guidelines. The sanitation program and personal hygiene requirements were implemented as the guidance given by suppliers and no further improvements were made. Moreover, the monitoring system design was neither tested for accuracy nor measured automatically e.g. using a portable thermometer. Calibration of the analytical equipment was on an ad-hoc basis, moreover, the task and frequency of calibration programs was unclear and not documented. Regarding the plan and design for sampling (e.g. microorganisms) was limited in microbiological expertise, lack of analysis facilities and strategies for improvement of the food safety and quality of processed products. *Pangasius* products were sampled based on experience and in-house knowledge and analysed by external laboratories without any checking by a third-party. The actual performance of the analytical equipment used (level 0) was not calibrated by the company itself nor by an external company. The low level of control activities in this company are correlated to the high levels of contamination found on the food contact surfaces, water and *Pangasius* samples. Therefore, the size of a company can indeed play a role in the further tailoring of the FSMS for certain activities such as sampling, microbiological analyses, maintenance and calibration (Sampers et al., 2012).

The core assurance activities were mostly assessed at level 1 (44% of the responses) up to level 2 (56% of the responses). Specifically, validation of the preventive measures and intervention systems were based on historical knowledge by the own company. The verification activities, documentation and record-keeping to support food assurance were performed on a regular basis and kept-up-to date in the documentation system (albeit not available online). As a result of less advanced control and assurance activities of the small company, the assigned score of 1_2 of FSMS was a lower level than required by the contextual factors (assigned score of 2_3).

In terms of external and internal food safety performance indicators (FSPI) in the large company, they sometimes dealt with ‘microbiological food safety complaints’, ‘hygiene related complaints’ and ‘hygiene and pathogen non-conformities’. From the interview it is mainly due to the sporadic occurrence of *L. monocytogenes* and *E. coli* on the final frozen product. It could be resolved when improvements in the company’s FSMS are made, e.g. optimizing the ‘hygienic design of equipment and facilities’, improving the ‘standard and tolerances design’, performing ‘experimental trials for company specific conditions’, etc. In the small company, the FSPI (assigned score 1_2) was in agreement with its FSMS. The

observation that the assurance activities are often lacking behind to control activities in FSMS, can for example be seen by the fact that auditing of the FSMS is performed yearly by one accredited third party and mostly based on historical knowledge. Therefore, this company often dealt with problems occurring in non-conformities, exceeding of microbiological guidelines and complaints by customers.

4.5 CONCLUSION

Although the overall MAS level of the large company was found to be slightly lower than the food safety self-assessment level, the actual performance of FSMS in this company regarding these microbiological enumerations is quite effective. However, it is taken into consideration for the standardization of the production processes in order to control the variability of microbiological quality well. In the small company, although the general microbiological quality of the final *Pangasius* products was acceptable from the point of view of the total aerobic psychrotrophic, *E. coli* and *S. aureus* counts, it was unacceptable with regards to some food safety parameters. The presence of hygiene indicators such as *E. coli* and *S. aureus* and the presence of *V. cholerae* on the hands of the food operators during processing, particularly in the packaging area, is a reflection of the poor personnel hygiene practices at the small scale processing plant evaluated in this study. From the results of the MAS combined with the self-assessment questionnaire of the quality operators, it can be suggested that the core control activities (i.e. hand hygiene, cleaning and disinfection) should be greatly improved in order to develop adequate cleaning and sanitation procedures for equipment, personnel and the processing environment.

Chapter 5 DECONTAMINATION OF *PANGASIUS* FISH WITH CHLORINE OR PERACETIC ACID IN THE LABORATORY AND IN A VIETNAMESE PROCESSING COMPANY

Redrafted from:

Tong Thi, A.N, Sampers I., Van Haute S., Samapundo, S., Nguyen, B.L, Heyndrickx, M., and Devlieghere, F., 2015. Decontamination of *Pangasius* fish (*Pangasius hypophthalmus*) with chlorine and peracetic acid in the laboratory and in a Vietnamese processing company. International Journal of Food Microbiology. Revision

ABSTRACT

This study evaluated the decontamination of *Pangasius* fillets in chlorine or peracetic acid treated wash water. The first part of the study evaluated the decontamination efficacy of the washing step with chlorinated water applied by a Vietnamese processing company during trimming of *Pangasius* fillets. Chlorine was only added at the beginning of the processing of a batch and was used continuously without renewal for 239 min. As a consequence, the total psychrotrophic counts, *Escherichia coli* and coliforms on the *Pangasius* fillets did not reduce significantly ($p > 0.05$) after washing at the Vietnamese company. This could be explained by the rapid accumulation of organic matter (*ca.* 400 mg O_2/L of COD after only 24 min) which resulted in a decrease in the level of free chlorine from 34.4 ± 2.9 ppm to 7.8 ± 3.6 ppm. In addition, the microbiological counts in the wash water increased to 5.7 (total psychrotrophic counts), 3.9 (coliforms) and 3.0 (*E. coli*) log CFU/100 ml after 24 min of washing. The second part of the study evaluated the disinfection efficacy of chlorine and peracetic acid (PAA) on both the wash water and *Pangasius* fillets on a laboratory scale. A single batch approach (one batch of wash water for treating a fillet) was used. Chlorine and PAA were evaluated at 10, 20, 50 and 150 ppm at contact times of 10, 20 and 240 s. Washing with chlorine and PAA wash water resulted in a reduction of *E. coli* on *Pangasius* fish which ranged from 0-1.0 and 0.4-1.4 log CFU/g, respectively while smaller reductions of total psychrotrophic counts, lactic acid bacteria and coliforms on *Pangasius* fish were observed. However, in comparison to PAA, chlorine was lost rapidly. As an example, 53-83% of chlorine and 15-17% of PAA were lost after washing for 40 s (COD = 238.2 ± 66.3 mg O_2/L). Peracetic acid can therefore be an alternative sanitizer. However, its higher cost will have to be taken into consideration. Where (cheaper) chlorine is used, the processors have to pay close attention to the residual chlorine level, pH and COD level during treatment for optimal efficacy.

5.1 INTRODUCTION

A crucial intervention step during the processing of frozen *Pangasius* fillets is washing to reduce the microbial loads on the food products as much as possible. Chlorine is the most commonly used disinfectant in fishery processing in general (Benjakul et al., 2012) and the processing of Vietnamese *Pangasius* fillets in particular (**Chapter 2**). Chlorine has been extensively studied for its effectiveness to inactivate vegetative bacteria on vegetables (López-Gálvez et al., 2010a; Lopez-Galvez et al., 2013), poultry (Bauermeister et al., 2008) and aquatic products (Benjakul et al., 2012; Kamireddy et al., 2008; Kim et al., 1999b). The antimicrobial effect of NaOCl mainly depends on the amount of free chlorine (in hypochlorous acid form, HOCl) present in the water, contact time and pH (optimal activity range and minimum corrosion of equipment = 6.5-7.5) (Fukuzaki, 2006; Suslow, 2008). A disadvantage is the rapid decomposition of chlorine through oxidation, addition, and electrophilic substitution reactions with organic substances in water (Van Haute et al., 2013a). In addition, the use of high chlorine concentrations may lead to the formation of excessive amounts of hazardous by-products such as trihalomethanes and chloramines (Alegria et al., 2009; López-Gálvez et al., 2010a). These concerns have led to the consideration of peroxyacetic acid or peracetic acid (PAA, CH_3CO_3H) as an alternative to halogenated disinfectants.

PAA is a peroxide of acetic acid, which is a stronger oxidant and disinfectant than either sodium hypochlorite. PAA has been used for disinfection of food contact surfaces, process water and aseptic packaging (González-Aguilar et al., 2012). The activity of PAA is less affected by organic matter or food material than chlorine (Kitis, 2004). No halogenated disinfection by-products have been observed after the treatment of environmental water with PAA (Monarca et al., 2002). However, a major disadvantage of PAA is its higher cost compared to NaOCl. In addition, in difference to the use of chlorine, the organic load of processing water is affected by the addition of PAA (López-Gálvez et al., 2009). Several studies have investigated the application of PAA to decontaminate vegetables (Vandekinderen et al., 2009a; Velde et al., 2013) and poultry (Bauermeister et al., 2008). To the knowledge of the authors, no studies have yet investigated the decontamination of fish with PAA.

In a previous study, it was noted that the use of a sanitizer in the wash water, specifically chlorine, did not significantly lower the microbiological counts on *Pangasius* fillets during processing in a Vietnamese company (**Chapter 2**). The low residual concentrations of

chlorine in the wash water used by Vietnamese companies and the high bacterial loads in the washing water as a function of time may explain this (**Chapter 4**). Therefore, the main objective of this study was to determine the disinfection efficacy of chlorine and (the alternative) PAA on both the wash water and the *Pangasius* fillets. First the microbiological build-up and the physicochemical properties of the wash water treated with chlorine during washing of *Pangasius* fillets at a Vietnamese processing plant were determined. In addition to this, the microbiological quality of the *Pangasius* fillets washed with this water were also determined. Subsequently chlorine and PAA were assessed with the aim of maintaining the microbial wash water quality on a lab-scale experiment. These results provide useful information that could be used to improve the microbial quality of *Pangasius* and related products in Vietnam.

5.2 MATERIALS AND METHODS

The **small scale company** that was evaluated in this study was located in Can Tho City. The characteristics of the sampled company have been described in **Chapter 2**.

5.2.1 Description of the washing process at the fillet trimming step

The full process flow diagram for the *Pangasius* fillets evaluated in this study is described in **Figure 4.1B** of **Chapter 4.** Washing was done at several points set in between the main processing steps such as filleting, skinning, trimming, and parasite control. During visits, fish after filleting and skinning were washed with tap-water while fish after trimming and checking parasite control were washed with chlorine for decontamination. Trimming itself was conducted in two steps (**Figure 5.1**). First, the subcutaneous fat and the red muscle was scraped-off (= trimming 1), followed by washing in tap-water (with a common ratio of product/water = 1/10) for 10 s to remove all muscle residues. During washing, tap-water (flow rate = *ca.* 3L/min) was run continuously to refill the washing tub. In the second part of trimming (= trimming 2), the belly fat, fins, bones, skins and tail of fish fillets were cut-off manually to achieve uniform and smooth fillets. The trimmed *Pangasius* fillets were then washed with chlorinated water combined with manual stirring. The chlorinated water was prepared by adding crushed-ice to tap water before dosing with chlorine. The initial concentration of chlorine in the chlorinated water was fixed at 50 ppm of NaOCl. This washing step was considered as the disinfection step. In practice, each batch of 10 kg of

Pangasius fillets were immersed in a water bath with 100 L of chlorinated water at 8 ± 4°C and stirred manually for *ca*. 10 s. Time between two batches was variable from 30 s to 5 min during visits, depending on production capacity, the products requirements, etc. (**Chapter 2**). The chlorinated water was prepared for use just before the shift started without adjustment of the pH. The chlorinated water was used for *ca.* 4 h without adjustment of the concentration, after which the washing tub was emptied and refilled with a new batch of chlorinated water.

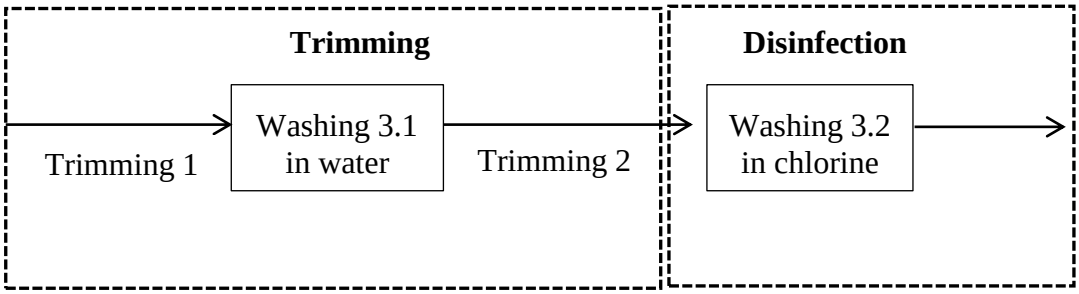


Figure 5.1 Procedure of trimming and washing step

5.2.2 Sampling and analysis of the **Pangasius** *fillets and wash water samples at a Vietnamese processing plant*

Pangasius fillets (*ca*. 200 g each) were sampled at the trimming (trimming 1 and trimming 2) and washing (in tap water and in chlorinated water) steps. The sampled fillets were aseptically taken with sterile tweezers and placed separately in sterile stomacher bags, which were then sealed and stored on ice until the microbiological analyses were done (within 5 h of sampling).

Water samples (*ca*. 500 ml) were collected aseptically during the washing step and stored in sealed sterile stomacher bags. The water samples were collected after 0, 10, 15, 24, 38, 60, 95, 151, and 239 min of use. Each sample was divided into three sub-samples. One of the sub-samples was aseptically transferred into a sterile Falcon tube containing 0.1M $Na_2S_2O_3$ (VWR, ProLao, China) as a neutralizing agent after which it was used for microbiological analyses. The water samples were stored on ice and transported in insulated boxes to the Laboratory of Microbiology and Biotechnology of Food Technology Department, Can Tho University, Vietnam for microbial analyses within 5 h of sampling. The second sub-sample was used to determine immediately the free and total chlorine by means of the HI93711 meter (Hanna Instruments, Temse, Belgium) based on N,N-diethyl-p-phenylenediamine (DPD) method (APHA, 1998). The third sub-sample was prepared for determination of the chemical oxygen

demand (COD). 10 ml aliquots of the water samples were transferred into a sterile Falcon tube which were frozen at -80°C and transported to the Laboratory of Food Microbiology and Biotechnology, Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University Campus Kortrijk, Belgium for analyses within 15 days.

The pH and temperature of the water samples were measured during sampling by means of a pH meter (Hanna Instruments, China) and a portable thermometer (± 0.1°C) (Multi-thermometer, China).

Upon arrival at the laboratory, a 25 g sample of fish sample was aseptically taken from different parts of each fillet by means of sterile scalpels and tweezers and placed in a sterile stomacher bag. 225 ml of sterile Maximum Recovery Diluent (MRD, Merck, Darmstadt, Germany) was then added, after which the mixture was homogenized for 1 min in a stomacher before serial (decimal) dilutions were prepared in test-tubes with 9 ml MRD. For the water samples, 1 ml of water was aseptically transferred to 9 ml MRD and was then vortexed for 10 s. Thereafter, a tenfold serial dilution series was performed. The total aerobic psychrotrophic counts (TPC) were determined on Aerobic Count Plate petrifilms™ (3M™ Microbiology Products, St. Paul, MN, USA) following incubation at 22°C for 72 h. Coliforms and *E. coli* were enumerated on coliform/*E. coli* petrifilms™ (3M™ Microbiology Products, St. Paul, MN, USA) after incubation at 37°C for 24 h and 48 h, respectively.

5.2.3 Evaluation of the efficacy of chlorine and peracetic acid as decontamination agents for **Pangasius** *fillets on a laboratory scale*

The efficacy of chlorine (50 g/L NaOCl, Javel, Horeca, Wommelgem, Belgium) and peracetic acid (PAA, Chriox 5, Christeyns NV, Ghent, Belgium), as decontamination agents was evaluated on a laboratory scale. Chriox 5, a stabilised mixture, consists of PAA (4.6-5%), hydrogen peroxide (23-25%), acetic acid (8-9%), water (60-65%) and stabilizer (<1%). Tap water was used as the control. Frozen *Pangasius* fillets (*Pangasius hypophthalmus*) were bought in a Belgian supermarket. The fillets varied in weight from 170 to 220 g including 10% glazing. The fillets were thawed by placing them at 2°C for 48 ± 2 h. Thawed fillets were then immersed individually in combination with manual stirring in water with or without disinfectant. A fish/water ratio of 1/10 was used, which is the same ratio used by the company evaluated in this study. For the treatment with chlorine, the pH of the wash water was

adjusted to 6.5 using 1 M hypochlorous acid. During washing, the temperature of the water was maintained at *ca.* 8 ± 2^{o}C.

The concentration of the decontamination agents was set at 10, 20, 50 and 150 ppm and three levels of the contact times of 10, 40 and 240 s were evaluated for each concentration. For each combination of concentration and contact time, three replicates were performed independently. In each independent repetition, all analyses were performed in duplicate.

5.2.3.1 Evaluation of the efficacy of microbial decontamination

After washing, both the fillet and wash water were sampled. 0.1 M $Na_2S_2O_3$ was used to neutralize chlorine whilst phosphate buffer (Na_2HPO_4 1.2g/L, NaH_2PO_4 0.22g/L, NaCl 8.5g/L, VWR, Prolabo, France) supplemented with sodium thiosulphate (1g/L) was used to neutralize PAA and catalase (500 mg/L, Sigma-Aldrich, Diegem, Belgium) was used to neutralize hydrogen peroxide residues present in PAA.

For fish samples, a 25 g composite sample was aseptically collected from different parts of the fillet and transferred into sterile stomacher bag. A ten-fold dilution was made in peptone water (Oxoid, Hampshire, U.K.) supplemented with the sterile neutralizing agents mentioned above. For the water samples, 10 ml of water was aseptically taken and transferred into sterile Falcon tubes containing the neutralizing agents. The fish and water samples were homogenized for 1 min and 10 s, respectively. Subsequently, a decimal dilution was made and enumeration was performed by pour-plating of 1 ml appropriate sample dilutions on specific media: Plate Count Agar (PCA, Oxoid, Hampshire, U.K.) incubated at 22°C for 72 h for total aerobic psychrotrophic counts, Rapid *E. coli* 2 Agar (Bio-Rad, Marnes-La-Coquette, France) incubated at 37°C for 24 h for *E. coli* and coliforms and de Man Rogosa Sharpe agar (MRS, Oxoid, Hampshire, U.K) incubated anaerobically at 22°C for 72 h for lactic acid bacteria. To determine the presence of *E. coli*/coliform in used water, 10 ml of water was pre-enriched for 24 h at 37°C in 90 ml of solution buffered peptone water (Oxoid, Hampshire, U.K.) supplemented with the sterile neutralizing agents mentioned above. Subsequently, this culture was streaked on Rapid *E. coli* 2 Agar (Bio-Rad, Marnes-La-Coquette, France) and incubated at 37°C for 24 h.

5.2.3.2 Physicochemical analysis

To avoid the interference of PAA or chlorine as a part of the organic matter in wash water, the chemical oxygen demand (COD) was only measured on the tap water samples according to the small-scale sealed-tube method (LCI 400; Hach Lange, Belgium). Turbidity was determined with a turbidimeter (HI98703; Hanna Instruments, Belgium); whilst chlorine (both free and total chlorine) and PAA were measured according to the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (Eaton et al., 2005) and an adjusted DPD method (Cavallini et al., 2013), respectively. The pH of the water was measured by a pH-electrode (Consort C380, Belgium).

5.2.3.3 Sensory evaluation

During the experiment, the sensory quality of the fish samples, general appearance, color, odor, and texture, was initially evaluated by two researchers. Thereafter, triangle tests were only conducted on the samples which were considered to be acceptable for human consumption. 19-22 panelists participated in the triangle tests. After washing in the sanitizers, the fillets were rinsed in tap water for 40 s before they were used in the sensorial tests. The sensory evaluation was performed on both fresh and cooked fish. Each panelist had to evaluate the color and texture of the fresh fish samples and the odor and color of the cooked fish samples. For the cooked samples, *ca.* 100 g of fish were placed in a closed glass jar for 4 min at 850W in a microwave. The heated samples were then cooled down for *ca*. 5 min before they were presented to the panelists. In all cases a set of three pieces of fish (each piece *ca.* 50 g), randomly coded with 3-digit numbers, were presented to panelists. The panelists were told about two of the pieces were similar and were asked to identify the odd sample. The panelists were also asked to specify the samples they preferred and if any of the samples were unacceptable for consumption. The number of correct answers were compared to the number expected by using a statistical table to achieve a significant answer (5% level) according to the BS ISO4120:2004 (ISO, 2004).

5.2.3.4 Color measurement

The color is defined by three orthogonal co-ordinates of L^*, a^*, b^*. L^* is the lightness component, which ranges from 0 (black) to 100 (white). The a^* (-green to + red) and b^* (-blue to +yellow) parameters both range from -120 to 120. A portable spectrophotometer (CM-

2500d, Konica Minolta Sensing Inc., Osaka, Japan) running on Spectra Magix^{TM} NX (Color Data software CM-S100w, Konica Minolta Sensing) software expressed in CIE L^*, a^*, b^* color space was used to measure these three color parameters. Each treatment described above was performed on 5 different pieces of fish fillets and the color of 3 randomly chosen points was measured on each fillet.

5.2.4 Statistical analysis

Results of the physicochemical characteristics and the microbiological analysis (log CFU/g or log CFU/100 ml) were reported as mean value ± standard deviation. Differences in the mean value of each treatment (time and concentration) were statistically assessed using a non-parametric Kruskal-Wallis H-type test due to showed non-normality. Thereafter, comparison of the paired means was done using the Mann-Whitney U test ($\alpha = 0.05$).

5.3 RESULTS

5.3.1 Evaluation of the washing steps at the Vietnamese **Pangasius** *processing company*

The total psychrotrophic counts (TPC) on the fillets sampled at trimming 1, tap-water wash, trimming 2 and chlorine-water wash did not differ significantly ($p > 0.05$) from each other. The same trend was observed for the *E. coli* and coliform counts on these samples (**Table 5.1**).

Table 5.1 Microbial counts on *Pangasius* fish during the trimming and washing steps

Process	Total psychrotrophic counts	Coliforms	*E. coli*
Trimming 1	$6.1 \pm 0.6^{a*}$	3.9 ± 0.7^{a}	2.6 ± 0.5^{a}
Water -wash	6.0 ± 0.5^{a}	4.1 ± 0.3^{a}	2.5 ± 0.3^{a}
Trimming 2	6.0 ± 0.4^{a}	4.0 ± 0.6^{a}	2.5 ± 0.4^{a}
Chlorine- wash	5.9 ± 0.3^{a}	3.9 ± 0.5^{a}	2.5 ± 0.5^{a}

*Data expressed as mean value ± standard deviation (log CFU/g) of at least 9 replicates. Means between the products sampled in this study indicated with the same superscript letter were not significantly different ($p > 0.05$)

The initial TPC counts, coliforms and *E. coli* of tap water used for washing after trimming 1 were 5.6 ± 0.6, 3.3 ± 1.0, 3.1 ± 0.5 log CFU/100 ml, respectively. This indicates that the wash bath was not disinfected properly before use. These counts of TPC, coliforms and *E. coli* increased over the first 24 min of use after which the levels remained relatively stable (**Figure 5.2**).

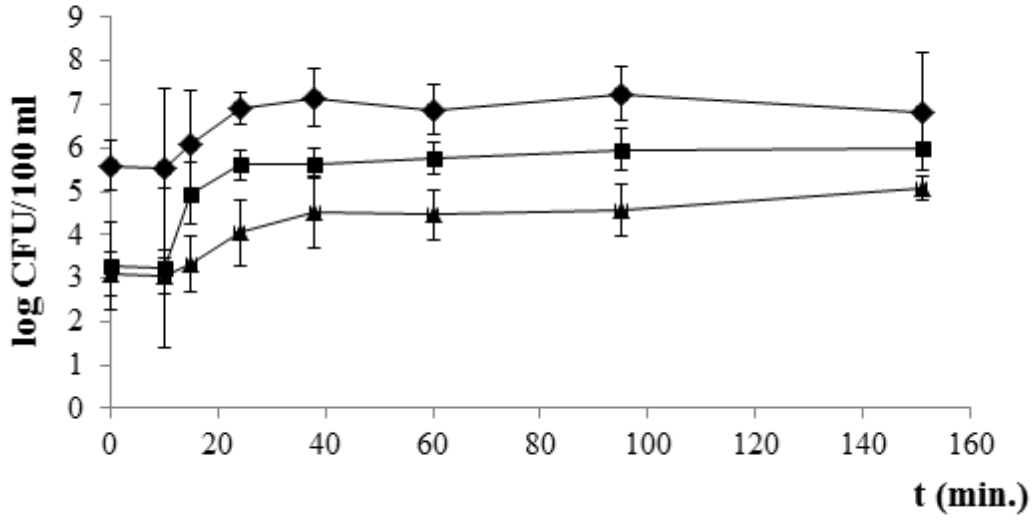


Figure 5.2 Microbial contamination of water in the washing tank during the washing of *Pangasius* fillet after trimming 1, ♦ Total psychrotrophic counts,■ Coliforms, ▲ *E. coli* Error bars denote standard deviation (n = 3). Quantification limit of 3 log CFU/100 ml

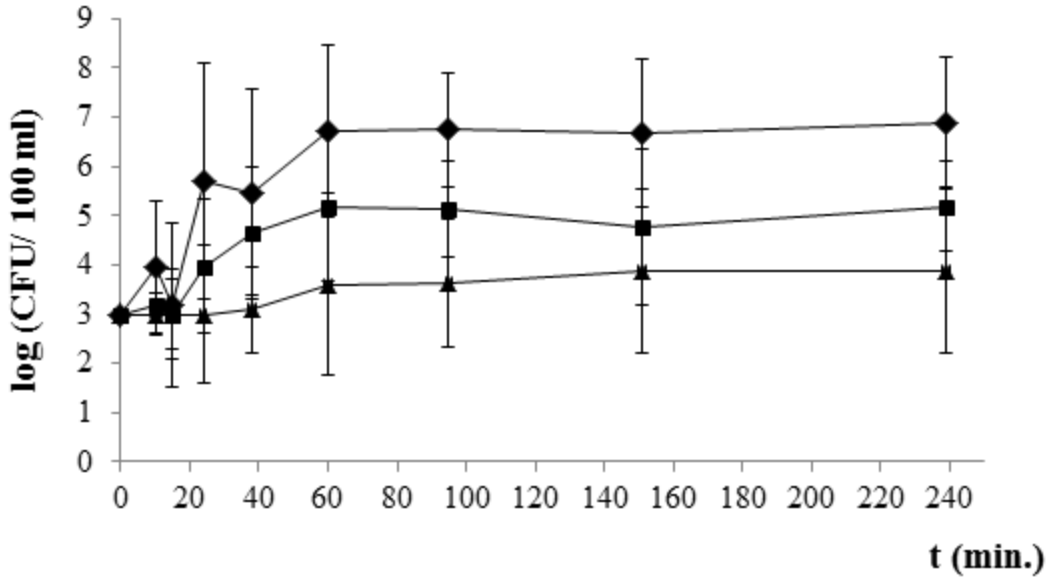


Figure 5.3 Microbial contamination of the chlorinated water in the washing tank during the washing of *Pangasius* fillet after trimming 2, ♦ Total psychrotrophic counts, ■ Coliforms, ▲ *E. coli*. Error bars denote standard deviation (n = 3). Quantification limit of 3 log CFU/100 ml

Unlike the wash water used after trimming 1, lower initial levels of total psychrotrophic aerobic counts (< quantification limit of 3 log CFU/100 ml) were found in the chlorinated wash water used after trimming 2 (**Figure 5.3**). As observed for the wash water after

trimming 1, a rapid increase in the microbial load of the chlorinated water occurred over the first 24 min of use after which the contamination levels remained stable.

The COD level increased gradually from 6.4 to *ca.* 1500 mg O_2/L after 239 min of washing. The opposite trend was observed for the levels of both total and free chlorine. The initial concentration of total and free chlorine was 40.8 ± 3.3 and 34.4 ± 2.9 ppm, respectively, and thereafter, a rapid decline was observed during the washing process. As an example, after 24 min of washing, the free chlorine level was only 7.8 ± 3.6 ppm and subsequently reduced to 0.7 ± 0 ppm after 239 min (**Figure 5.4**).

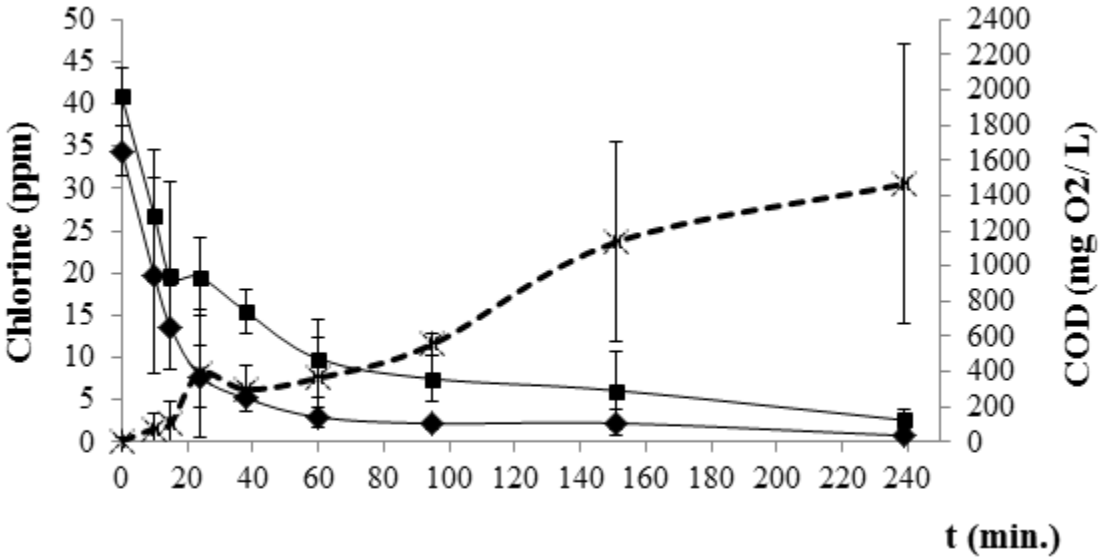


Figure 5.4 Total chlorine (■), free chlorine (♦) and COD (∗) of chlorinated water during the washing of *Pangasius* fillet after trimming 2. Error bars denote standard deviation (n = 3)

The pH values of the wash water without chlorine and with chlorine were similar at 7.8 ± 0.3 and 7.4 ± 0.2, respectively. The temperature of the wash water without chlorine and with chlorine was 29.8 ± 0.6°C and 6.3 ± 2.2°C, respectively.

5.3.2 Evaluation of physicochemical parameters of wash water on a laboratory scale

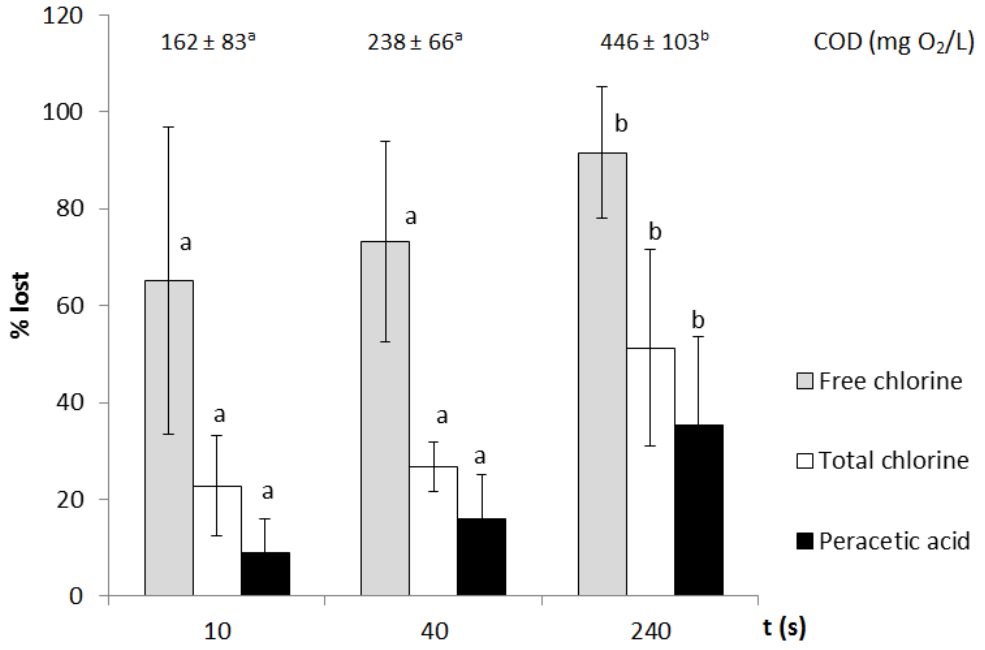


Figure 5.5 Percentage of the loss of sanitizers during washing in function of COD (related to time that the fillet was immersed) performed in a laboratory scale. Error bars denote standard deviation of at least three replicates. Value with different superscript letter between time of treatment shows statistical significance.

The degradation of both disinfectants (chlorine and PAA) and COD accumulation was independent of the initial concentration ($p > 0.05$) but dependent on duration of the treatment ($p < 0.05$). The concentrations of free chlorine in the wash water targeted at 10, 20, 50 and 150 ppm were actually 9.5 ± 0.4, 18.0 ± 1.0, 44.7 ± 3.4 and 141.3 ± 9.4 ppm, respectively. During washing, the quantity of free chlorine lost after exposure times of 10 s (65.1 ± 31.7% of loss) and 40 s (73.3 ± 20.7%) were not significantly different ($p > 0.05$), whilst the losses after 10 and 40 s were determined to be significantly different ($p < 0.05$) to those after 240 s of exposure (91.6 ± 13.5%). The actual concentrations of PAA in wash water targeted at 10, 20, 50 and 150 ppm were 9.7 ± 0.3, 19.6 ± 0.8, 46.1 ± 1.9 and 142.7 ± 3.7 ppm, respectively. The degradation of PAA increased with treatment time of 10 s (9.0 ± 7.1% of degradation), 40 s (15.9 ± 9.3%) and 240 s (35.2 ± 18.3%) (**Figure 5.5**).

Turbidity was determined to increase in function of increasing COD. No significant difference in turbidity was seen after 10 s and 40 s of washing, in tap water, chlorinated water and PAA

water (**Figure 5.6**). However, after 240 s of washing, the turbidity of the chlorinated wash water (21.7 ± 6.9 NTU, Nephelometric Turbidity Unit) was significantly higher ($p < 0.05$) than that of tap water (11.6 ± 5.1NTU) and PAA water (17.4 ± 23.4 NTU). No significant differences ($p > 0.05$) were found between the turbidities of tap water and PAA water. A large variation, ± 23.4 NTU, was observed in wash water with PAA sampled after 240 s.

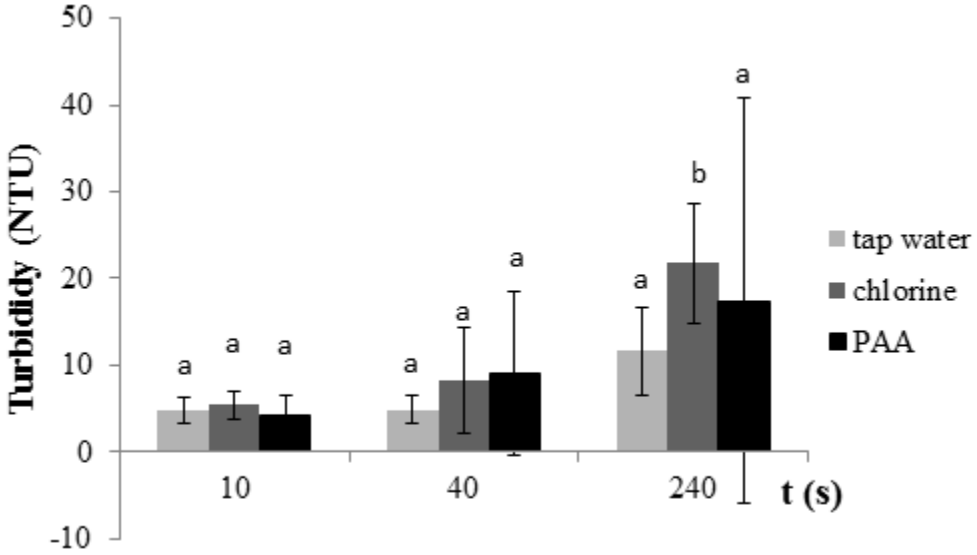


Figure 5.6 Turbidity of wash water during treatment time. Data are expressed as mean value ± standard deviation (NTU) of at least three replicates. Value with different superscript letter between sanitizers in the same groups (time of treatment) shows statistical significance.

The temperature of the wash waters was 8.6 ± 1.8°C. The initial pH of the tap water ranged from 7.8 to 8.1. After washing for different times, the pH of PAA wash water, chlorinated wash water and tap water ranged from 5.7-7.8, 6.7-7.9 and 7.2-8.3, respectively.

5.3.3 Evaluation of effect of water sanitizers on the microbiological quality of wash water on a laboratory scale

The counts for all the bacteriological parameters evaluated of the fresh tap water used in this study were below the quantification limit (<3 log CFU/100 ml). **Table 5.2** shows the microbial loads in the wash water as a function of the treatment time. Increasing in the concentration of chlorine in the wash water resulted in a significant reduction ($p < 0.05$) of the TPC in the water samples. The reduction of TPC in chlorinated water was determined to be

less influenced by time of exposure. Between 10 s and 40 s of treatment, the levels of TPC in the chlorinated wash water at a particular concentration of chlorine did not differ significantly ($p > 0.05$). However, at 10 and 50 ppm of chlorine, the TPC of the chlorinated water after 240 s of washing were significantly higher ($p < 0.05$) than those found after 10 s and 40 s of washing. For example, in wash water with 50 ppm chlorine, the TPC were 4.9 ± 0.2, 3.9 ± 0.8 and 3.6 ± 0.8 log CFU/100 ml after 240 s, 40 s, and 10 s, respectively.

Similar results were observed in PAA wash water. Significantly lower ($p < 0.05$) TPC were found in wash water with higher concentrations of PAA. No significant differences ($p > 0.05$) were found between the TPC of the wash water with 10, 20 and 150 ppm PAA after exposure for 10, 40 and 240 s. An exception was found at 50 ppm, where the TPC in the PAA wash water was determined to be significantly different ($p < 0.05$) after 40 s (4.3 ± 0.1 log CFU/100 ml) and 240 s (3.8 ± 0.1 log CFU/100 ml) of exposure. The counts after an exposure of 10 s (4.1 ± 0.4 log CFU/100 ml) in the wash water with 50 ppm of PAA did not differ significantly ($p > 0.05$) with the counts after exposure for 40 s and 240 s (**Table 5.2**).

The lactic acid bacteria (LAB) counts in the tap water after washing for 10, 40 and 240 s did not differ significantly ($p > 0.05$) from each other. The profiles (trends) of the LAB counts in chlorinated and PAA wash water were similar to those of TPC. A significant reduction ($p < 0.05$) of the LAB counts occurred with an increase in concentration of both chlorine and PAA, while no significant reduction ($p > 0.05$) of that occurred with an increase in exposure times (**Table 5.2**). The only exceptions were the significant increase ($p < 0.05$) of the LAB counts in the wash water with 10 and 50 ppm chlorine occurred with an increased exposure times.

Table 5.2 Microbial wash water contamination (log CFU/100 ml) during the decontamination of *Pangasius* fillets

Log CFU/ 100 ml	Concentration (ppm)	Total psychrotrophic counts			Latic acid bacteria			Coliforms			*E. coli*		
		Time (s)											
		10	40	240	10	40	240	10	40	240	10	40	240
Tap water		$5.3 \pm 0.1^{1*}$	5.4 ± 0.1^{2}	5.1 ± 0.8^{12}	3.6 ± 0.2^{1}	3.8 ± 0.2^{1}	4.1 ± 0.4^{1}	<3.0	3.2 ± 0.2	3.3 ± 0.2	<3.0	<3.0	<3.0
Chlorine	10	4.9 ± 0.5^{b1}	5.3 ± 0.2^{ab1}	5.7 ± 0.0^{c2}	3.1 ± 0.1^{a1}	3.7 ± 0.5^{b12}	3.9 ± 0.3^{c2}	<3.0	<3.0	3.4 ± 0.5	<3.0	<3.0	<3.0
	20	4.6 ± 0.6^{ab1}	4.8 ± 0.6^{bc1}	4.5 ± 1.3^{ab1}	3.0 ± 0.1^{a1}	3.0 ± 0.1^{a1}	3.4 ± 0.4^{abc1}	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
	50	3.6 ± 0.8^{ab1}	3.9 ± 0.8^{ac1}	4.9 ± 0.2^{b2}	$<3.0^{a2}$	$<3.0^{a2}$	3.3 ± 0.2^{b1}	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
	150	3.4 ± 0.6^{a1}	3.1 ± 0.2^{a1}	3.2 ± 0.4^{a1}	$<3.0^{a1}$	$<3.0^{a1}$	$<3.0^{a1}$	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PAA	10	5.1 ± 0.2^{b1}	4.9 ± 0.2^{b1}	5.0 ± 0.2^{ab1}	3.5 ± 0.2^{b1}	3.3 ± 0.3^{a1}	3.1 ± 0.2^{a1}	< 3 (+**)	< 3 (+)	< 3 (+)	< 3 (-)	< 3 (-)	< 3 (-)
	20	4.7 ± 0.1^{a1}	4.8 ± 0.4^{ab1}	4.6 ± 0.1^{b1}	3.1 ± 0.2^{ab1}	3.4 ± 0.4^{a1}	3.1 ± 0.2^{a1}	< 3 (+)	< 3 (+)	< 3 (+)	< 3 (-)	< 3 (-)	< 3 (-)
	50	4.1 ± 0.4^{ab12}	4.3 ± 0.1^{a2}	3.8 ± 0.2^{a1}	$<3.0^{a1}$	$<3.0^{a1}$	$<3.0^{a1}$	< 3 (+)	< 3 (+)	< 3 (+)	< 3 (-)	< 3 (-)	< 3 (-)
	150	3.0 ± 0.0^{ab1}	3.7 ± 0.7^{a1}	3.0 ± 0.0^{ab1}	$<3.0^{a1}$	$<3.0^{a1}$	$<3.0^{a1}$	< 3 (+)	< 3 (+)	< 3 (+)	< 3 (-)	< 3 (-)	< 3 (-)

*Data are expressed as mean value ± standard deviation (log CFU/100 ml) of at least three replicates. Quantification limit = 3 log CFU/100 ml.

** Presence (+) or Absence (-) (of 10 ml water samples) by an enrichment procedure.

Different superscript letters between various concentrations in the same column show statistical significance ($p < 0.05$).

Different superscript numbers between time treatments in the same row show statistical significance ($p < 0.05$).

The counts of coliforms and *E. coli* in the chlorinated and PAA wash water were in most cases lower than the quantification limit (<3 log CFU/100 ml). No *E. coli* occurred in 10 ml of PAA wash water while sample enrichment showed that coliforms survived at all time and concentration combinations. Enrichment was not performed on the wash water samples originating from fillets washed with chlorinated water (**Table 5.2**).

5.3.3.1 Evaluation of the effect of water sanitizers on the microbial quality of Pangasius fillets on a laboratory scale

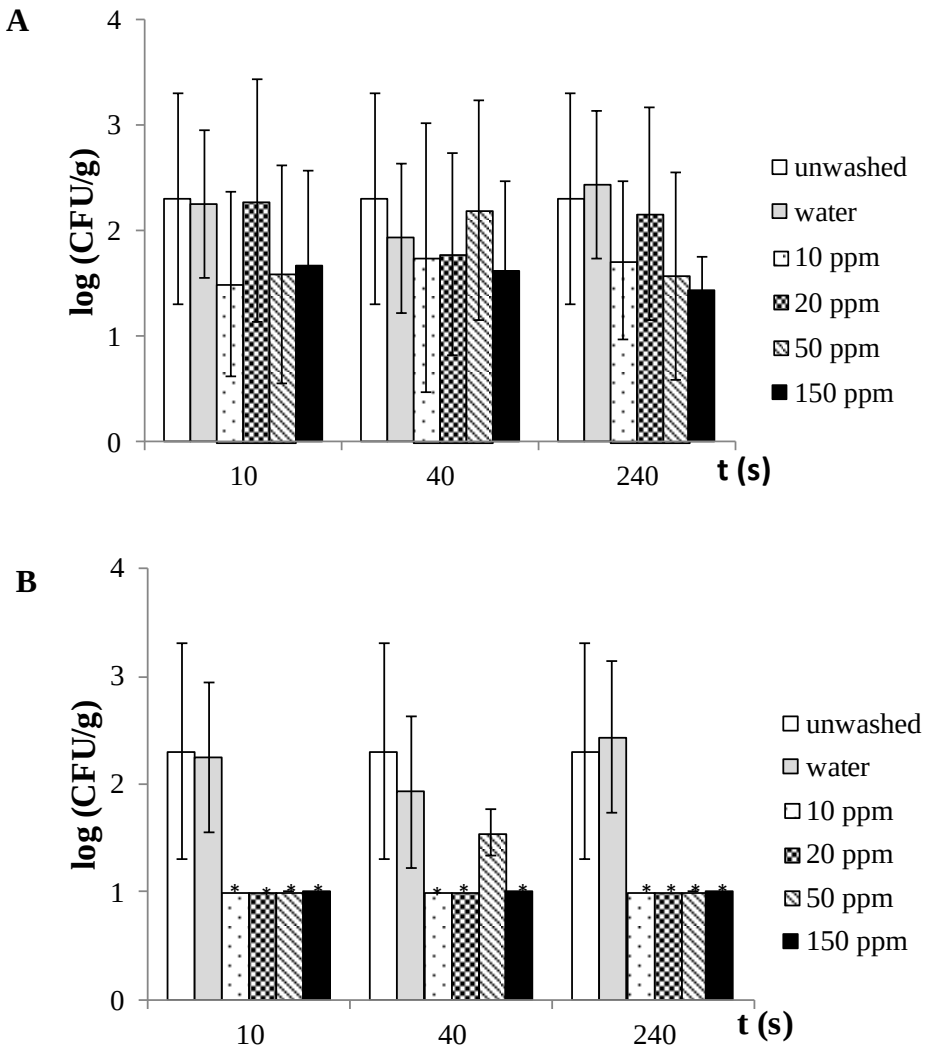


Figure 5.7 *E. coli* counts of *Pangasius* treated with chlorine (A) and peracetic acid (B), ***** indicates values below or equal to detection limit (≤ 1 log CFU/g)

The antimicrobial effect of chlorine and PAA sanitizers was evaluated on *Pangasius* fillets. The initial TPC, *E. coli*, coliforms and LAB counts of unwashed *Pangasius* fillets were 5.6 ±

0.3, 2.4 ± 0.7, 3.5 ± 0.3 and 4.1 ± 0.3 log CFU/g, respectively. The reduction of TPC on the *Pangasius* fillets washed in chlorinated and PAA water ranged 0-0.3 log CFU/g and 0-0.1 log CFU/g, respectively. The LAB counts on the *Pangasius* fillets were reduced by 0-0.4 and 0-0.5 log CFU/g when they were washed in chlorinated and PAA water, respectively. The coliforms on *Pangasius* fillets were reduced by 0 to 0.3 log CFU/g when the fillets were washed with chlorine water compared to 0 to 0.4 log CFU/g when the fillets were treated with PAA wash water. The lower reduction was observed for *E. coli* by 0-1.0 log CFU/g on the *Pangasius* fillets washed in chlorinated wash water compared to a reduction of 0.4-1.4 log CFU/g on the *Pangasius* fillets washed in PAA wash water (**Figure 5.7**).

5.3.3.2 Sensory analysis

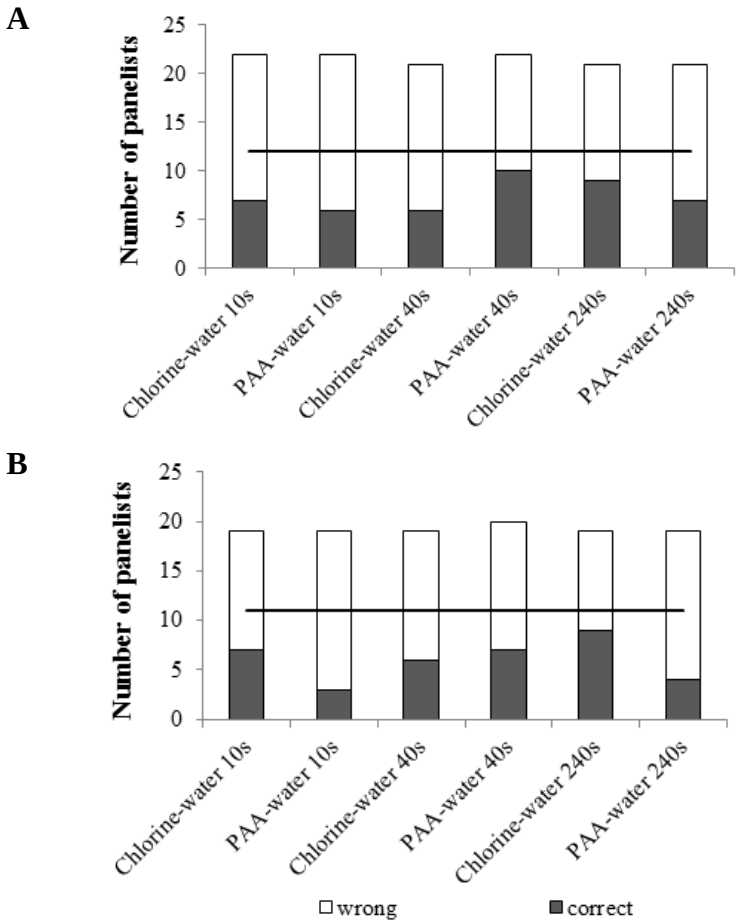


Figure 5.8 Sensorial test of raw (A) and cooked *Pangasius* (B) after washing in both chlorinated and peracetic acid water at 50 ppm. □ number of panelists choosing the wrong samples as the odd sample, ■ number of panelists choosing the correct samples as the odd sample. The line shows a minimum number of correct answers to have a significant difference in sensory quality between series ($\alpha = 0.05$).

The sensorial quality of the fillets treated in water with 10 or 20 ppm of both chlorine and PAA at different treatment times, did not differ to that of fillets washed in tap water. This is in contrast with washing in 50 and 150 ppm chlorine or PAA which resulted in some changes to the sensorial properties of the fillets when they were compared with the fillets washed in tap water. Washing in 150 ppm of chlorine and PAA resulted in fillets that were discolored, with chlorine odors and soft texture after treatment. The sensorial changes after washing in 50 ppm chlorine and 50 ppm PAA were evaluated by means of triangle tests employing 19-22 assessors. It was determined that the overall quality of the fillets (color, texture and odor) washed in 50 ppm chlorine and PAA did not differ significantly ($p > 0.05$) from those washed in tap water (**Figure 5.8**).

5.3.3.3 Effect of washing in chlorine and PAA on the color of Pangasius *fillets*

In addition to the sensory evaluation, changes in the L^*, a^* and b^* color parameters on *Pangasius* fillets as a result of washing in 50 ppm chlorine or PAA at 50 ppm after contact times of 10, 40 and 240 s were determined (**Table 5.3**). Washing in either 50 ppm chlorine or 50 ppm PAA had no significant effect ($p > 0.05$) on the L^*, a^* and b^* values in comparison to L^*value of fillets washed in tap water. The only exceptions were significantly lower ($p < 0.05$) a^* and b^* values observed on *Pangasius* fillets washed for 240 s in chlorinated and PAA water, respectively compared to that washed in tap water for the same duration.

Table 5.3 Effect of chlorine (50 ppm) and peracetic acid (50 ppm) treatment on the color of *Pangasius* fillets

Samples	**Time (s)**	L^*	a^*	b^*
Chlorine	10	$53.6 \pm 3.7^{a1*}$	-1.3 ± 0.5^{a}	4.6 ± 1.1^{a}
water		56.8 ± 6.3^{a}	-1.1 ± 0.9^{a}	4.8 ± 2.7^{a}
PAA	10	56.2 ± 4.5^{a}	-2.9 ± 0.4^{a}	4.8 ± 1.0^{a}
water		58.5 ± 5.0^{a}	-2.8 ± 0.5^{a}	4.2 ± 1.1^{a}
Chlorine	40	54.7 ± 2.1^{a}	-2.3 ± 0.6^{a}	6.4 ± 1.5^{a}
water		54.0 ± 3.7^{a}	-2.1 ± 0.7^{a}	6.4 ± 1.4^{a}
PAA	40	56.3 ± 3.9^{a}	-1.9 ± 0.8^{a}	5.4 ± 1.8^{a}
water		54.0 ± 3.7^{a}	-2.1 ± 0.7^{a}	6.4 ± 1.4^{a}
Chlorine	240	54.9 ± 5.5^{a}	-2.8 ± 0.4^{a}	5.0 ± 2.2^{a}
water		52.8 ± 3.2^{a}	-2.1 ± 0.6^{b}	5.9 ± 1.3^{a}
PAA	240	57.3 ± 3.7^{a}	-1.7 ± 0.6^{a}	3.7 ± 1.2^{a}
water		55.5 ± 5.6^{a}	-1.6 ± 0.6^{a}	4.9 ± 1.8^{b}

[1]Data are expressed as mean value ± standard deviation of five replicates

[*]Different superscript letters between two samples in the same column show statistical significance ($p \leq 0.05$)

5.4 DISCUSSION

5.4.1 Efficacy of the disinfection protocol used at the Vietnamese processing company

Chlorinated water was used during processing at the Vietnamese company investigated in this study to decontaminate *Pangasius* products. This has been discussed in our previous studies (**Chapter 2** & **Chapter 4**). Those studies determined that the use of chlorine had no effect on microbial quality of *Pangasius* products processed at this company. Potential reasons were suggested due to the inappropriate application of the chlorine such as the chlorine concentration, pH, and organic matter of the chlorinated water. These factors were overlooked by the processor. The results of this study confirmed that the suggested reasons were responsible for the observed lack of efficacy. The concentration of free chlorine decreased rapidly, from 34.4 ± 2.9 ppm to 7.8 ± 3.6 ppm after 24 min of use. The organic matter in the wash water rose rapidly, *ca.* 390 mg O_2/L of COD was detected after only 24 min of washing. In addition, the pH of the chlorinated water (7.8 ± 0.3), was outside the range for the optimal activity of chlorine (pH 6.5-7.5). Some previous studies have determined that chlorine is much more effective for inactivation of bacteria in wash water than it is for decontamination of products surfaces (Gil et al., 2009; Sapers, 2001). The quality of wash water is important for the effectiveness of the washing process and its quality deteriorates rapidly as a result of the accumulation of product residues and other foreign materials (Ragaert et al., 2007). In addition, a high risk of contamination of the *Pangasius* fillets by *Vibrio cholerae* was determined to occur during washing in tap water at the same company (**Chapter 4**). Therefore, the use of sanitizers to reduce or eliminate the microbial loads in the water is necessary.

However, it is essential that enough active sanitizer is maintained in the wash water to avoid cross-contamination between products from different batches or between products in the same batch as has been emphasized in the studies performed on vegetables (Allende et al., 2008; López-Gálvez et al., 2009; Van Haute et al., 2013d). In this study, the high TPC counts seen during the initial stages of washing (*ca.* 5.5 log CFU/100 ml) may have been caused by the inadequate hygiene and sanitation levels at the sampled company (**Chapter 4)**. The bacterial contamination levels in the wash water increased gradually as a function of time during washing due to accumulation of microorganisms and large amounts of nutrients from suspended matter and protein (Mameri et al., 1996; Ragaert et al., 2007). A large quantity of fish was washed in a water bath (*ca.* 620 kg fillets/151 min) and in a bath with chlorinated

water (*ca.* 750 kg/239 min, data not shown) before the water was renewed. In this way, there is a high probability of transmission of microorganisms from contaminated products into the wash water (Allende et al., 2008). Chlorine played an effective role in maintaining low microbial loads in the chlorinated water during the first 10-15 min of washing (TPC counts ranged 3.2-4 log CFU/100 ml after 10-15 min of washing). However, the chlorinated water was used for up to 4 h at the company. Increase in the the microbial counts in the chlorinated water started after 15 min of washing (e.g. TPC increased up to 5.7 log CFU/100 ml after 24 min of washing). It has been determined that the buildup of the microbial load in wash water directly influences the quality and safety of the washed products (Allende et al., 2008). Therefore, it is essential that enough residual chlorine remains in the chlorinated water by continuously adding to prevent further contamination (Casani et al., 2005; Van Haute et al., 2013a). The optimum residual level of chlorine in wash water used for *Pangasius* products should be established to improve the efficacy of decontamination.

5.4.2 Disinfection efficacy in lab scale experiment

Pangasius fillets were washed in chlorine or PAA water in the lab, with washing in tap water as the reference treatment. Higher reduction of the TPC in the wash water was observed when high concentrations of chlorine were used. Although in general the reduction of the TPC in chlorinated water was less influenced by time of exposure, a longer treatment time resulted in an increase in the bacterial counts in the wash water i.e. the TPC increased from 3.9 ± 0.8 to 4.9 ± 0.2 log CFU/100 ml in 50 ppm chlorine water after it had been used for 40 and 240 s, respectively. In this study, active free chlorine was lost rapidly (53-83% of loss after 40 s and 82-98% after 240 s) after a single batch was washed. Meanwhile, a significant increase of the COD and turbidity was seen during time of treatment. Increase in the COD was related to the loss of active free chlorine since chlorine reacts with organic matter. This accounts for the limited efficacy of chlorine in maintaining the microbial quality of water when it is used in a batch washing system. The levels of decontamination of the *Pangasius* fillets were also investigated. The initial counts on the *Pangasius* fillets sampled in this study were in agreement with previous studies regarding the microbiological quality of Vietnamese *Pangasius* products (**Chapter 2** & **Chapter 4**). Chlorine was limited in its ability to reduce the microbiological counts on *Pangasius* fillets as the maximum reductions observed were 0.3 (TPC), 0.4 (LAB), 0.3 (coliforms) and 1.0 log CFU/g (*E. coli*) in this study. Some previous

studies on poultry and vegetable indicated that the efficacy of the antimicrobials used is influenced by the level of bacterial attachment, initial counts of microorganisms on the products, and susceptibility of microorganisms to the antimicrobials (Bauermeister et al., 2008; Jahid and Ha, 2012; Takeuchi and Frank, 2001; Van Haute et al., 2013d). Moreover, because treatment with disinfectants can affect the sensory properties of fish, it is important to determine potential changes in the overall sensory quality of *Pangasius* treated with chlorine. In this study, the organoleptic properties of the chlorine-treated *Pangasius* were largely satisfactory. The only exception was fish washed with 150 ppm chlorine water which was discolored and had a chlorine odor (especially notable after ≥ 40 s). As this study was limited to evaluation of chlorine at 0, 10, 20, 50 and 150 ppm, the acceptability of the organoleptic properties of chlorine-treated *Pangasius* should be further evaluated at concentrations between 50 and 150 ppm. On the other hand, some studies on vegetables indicated that low concentrations of chlorine could be used to wash products without loss of antimicrobial efficacy whilst reducing the risk of formation of toxicity of chlorination by-products (López-Gálvez et al., 2009; Luo et al., 2011; Van Haute et al., 2013a).

The use of PAA as an alternative sanitizer has been sporadically studied for washing vegetables, fruits and poultry (Bauermeister et al., 2008; Vandekinderen et al., 2009b; Velde et al., 2013). In this study PAA was less influenced by the presence of organic matter as the concentration of PAA residues decreased to a less extent during treatment (15.9 ± 9.3% of loss after 40 s). The high PAA residue concentration in the water reduced the microbial loads in water sampled in this study. PAA was also very effective against *E. coli* because no *E. coli* was recovered from 10 ml samples of PAA water irrespective of the combination of concentration and contact time. However, coliforms were still recovered when the water samples were enriched. This indicates that *E. coli* is more sensitive to PAA than others species of coliforms. In contrast, reduction of LAB on *Pangasius* fillets was maximally 0.5 log CFU/g compared to 1.4 log CFU/g for *E. coli* reduction. Gram-positive bacteria are less sensitive than Gram-negative bacteria. Ruiz-Cruz et al. (2007) showed that the reduction of bacteria inoculated onto shredded carrots by PAA (40 ppm) was ranked as follows in decreasing order of efficacy: *Salmonella*, *E. coli* O157:H7 (both Gram negative) and *L. monocytogenes* (Gram-positive). It should be kept in mind that PAA was applied in this study on a single batch without reuse. Therefore, further studies should evaluate washing with PAA in a continuous system as applied at the Vietnamese company investigated in the study.

In similarity to the results observed for chlorine, the sensorial properties of fish washed in 150 ppm PAA were changed after treatment. This was characterized by a typical PAA odor, a soft texture and surface discoloration; however, the acceptability of *Pangasius* treated in PAA water of 150 ppm should further tested. Application of PAA at lower concentrations (≤ 50 ppm) did not significantly affect the sensorial quality of the fish. Although no significant differences were determined by means of the Triangle test between either fresh and cooked *Pangasius* washed with water of 50 ppm PAA for 240 s, the b^* value of fish treated with PAA was significantly lower ($p < 0.05$) than that of fish washed in tap water. This indicated a yellowness decrease. Although the L^* parameter was higher in the PAA treated fish, indicating a lighter color, these values were not significantly different ($p > 0.05$) from those of the fillets washed in tap water.

5.5 CONCLUSION

To our knowledge, this is the first study performed specifically on the washing step applied to Vietnamese *Pangasius* products destined for export. Chlorine was not used properly by the company that was investigated. It was only added at the beginning of the batch and due to the very long application time of the chlorine washing water without renewal (239 min), a high possibility actually existed of cross contamination taking place from the contaminated water to the fish. The use of a single batch of chlorinated water for a batch of fish or continuous adding of chlorine to maintaining wash water disinfection capacity can be used to improve the level of decontamination attained on the fish as well as to avoid the possibility of cross contamination taking place from the wash water to products. In general, the microbial quality of the wash water and fish after treatment with chlorine was similar to that treated with PAA. However, PAA water can be used for a longer time (or over more batches) as its efficacy is less influenced by the certain accumulation of organic matter during treatment. Therefore, preserving the chlorine concentration is essential in case of implementing chlorine in continuous washing processes.

Chapter 6 EVALUATION OF THE SAFETY AND QUALITY OF WASH WATER DURING THE BATCH WASHING OF *PANGASIUS* FISH IN CHLORINATED AND NON-CHLORINATED WATER

Redrafted from:

Tong Thi, A.N, Sampers I., Van Haute S., Samapundo, S., B. D. Meulenaera, Heyndrickx, M., and Devlieghere, F., 2015. Evaluation of the safety and quality of wash water during the batch washing of *Pangasius hypophthalmus* in chlorinated and non-chlorinated water. Under preparation.

ABSTRACT

Disinfection of *Pangasius* by washing in chlorinated water is commonly implemented during processing. Chlorine is often added at the beginning of the batch and continuously used for washing several batches. However, the disinfection efficacy of chlorine decreases in the presence of organic matter and it also degrades very rapidly. To improve the microbial quality of the wash water and in turn to avoid the cross contamination from the wash water to the products, this study investigated the continuous dosing of chlorine to the wash water by means of a pump to maintain antimicrobial levels of free chlorine in the washing bath during the washing of 12 batches of *Pangasius* fillets. In comparison to the control (no chlorination), a reduction in the microbial counts of the chlorinated wash water was observed. As an example, the counts of psychrotrophic bacteria reduced by between 2-4 log CFU/100 ml of wash water, but no reducing effect on the fish fillets was found. After the final batch was washed, total chlorine and organic matter in the water sampled had accumulated to 482.9 ± 17.0 ppm and 4447.5 ± 187.4 mg O_2/L, respectively. However, only 8.9 ± 1.3 µg/L of trihalomethanes were formed in the chlorinated wash water whereas no trihalomethanes were detected in the washed *Pangasius* fillets after rinsing. It was concluded that the *Pangasius* fillets washed in chlorinated water continuously dosed with chlorine were safe for human consumption even after 12 batches had been washed. However, the organoleptic properties of *Pangasius* fillets washed in the final batch were unacceptable due to discoloration (bleaching) and chlorine odor. The organic matter and residues of free chlorine in the wash water can be estimated more rapidly and conveniently by measuring UV absorbance at 249 nm and the oxidant redox potential (ORP), respectively.

6.1 INTRODUCTION

In the fishery industry, chlorinated water is used for different purposes including disinfecting the facilities, equipment, utensils (knives, cutting boards, baskets), workers' protective clothing (gloves and boots), hands and the fish surface. The dose of chlorine used varies depending on the intended use i.e. 50-200 ppm is used for washing floors and cleaning boxes and tables (Arbor, 2008), 50-100 ppm for disinfecting hands (Kanduri and Eckhardt, 2008), 50 ppm for disinfecting headless-shell shrimps (Arbor, 2008). Over the last decade, only a few studies have reported about the inactivation by chlorinated water of artificially inoculated pathogens onto fish surface (**Chapter 1**). Up to date, no studies have been reported regarding the decontamination of the natural microbiota of fish by chlorine.

Pangasius fish is washed in chlorine water (2-10 ppm) during processing in Vietnam (Anh et al., 2010) but no specific study has been performed on its effectiveness. Previously, it is reported that no significant differences occurred between the microbial evolution on *Pangasius* fish treated with non-chlorinated water and that treated with 50-80 ppm chlorine (**Chapter 2**). A further study evaluating the microbial safety and quality of *Pangasius* fish during processing reported no effective decontamination of fish manually washed in chlorinated water (50 ppm chlorine) (**Chapter 4**). Loss of the bactericidal activity chlorine used for washing the fish occurred as the chlorine was added once only at the start of the shift and this wash water was reused for 239 min. The accumulation of high microbial loads in reused wash water directly impacts the microbial quality of the washed products as a result of the transmission of microorganisms to the products (Allende et al., 2008) & (**Chapter 4**). On the other hand, chlorine decomposes very rapidly as a result of its reaction with organic matter (Suslow, 2008; Van Haute et al., 2013d). Therefore, in the industrial processes, it is more realistic to maintain a low level of chlorine in a washing bath sufficient to avoid cross contamination onto washed products i.e. for disinfection of the fish by a washing process. Cross contamination has been reported to be controlled by maintaining a residual chlorine level of at least 1 ppm after an hour during the washing of fresh cut lettuce (Van Haute et al., 2013a) whilst maintaining chlorine at 7 ppm has been reported to keep the wash water free from pathogens for 1 hour during the washing of fresh–cut spinach (Gómez-López et al., 2014). However, the use of chlorine may lead to the formation of disinfection by-products when organic matter is present in the water (FAO/WHO, 2008; Luo et al., 2011). These by-products include trihalomethanes (THMs), haloacetic acids, haloketones, haloacetinitriles,

chloral hydrate and chloropicrin (COT, 2006). THMs (including chloroform, bromodichloromethane, chlorodibromoethane and bromoform), haloacetic acids (including trichloroacetic, dibromoacetic and dichloroacetic acid) and trichloroacetaldehyde are possibly carcinogenic to humans (Gopal et al., 2007).

Some recent studies (mostly on fresh-cut vegetables) have also evaluated the chemical safety of both washed products and the washing water when disinfectants are used (Gómez-López et al., 2014; López-Gálvez et al., 2010a; Van Haute et al., 2013a). To date no study has been performed on the decontamination efficacy of both fish and wash water in a dynamic system. In this study, a surface decontamination process for *Pangasius* products in Vietnam was evaluated whereby successive batches of fish were washed in a bath where the chlorine concentration was maintained by continuously adding chlorine to the wash water by means of a pump. The residual chlorine levels and the accumulation of organic matter in the wash water were evaluated. The microbial inactivation in both the wash water and on the fish was investigated. In addition, the sensorial quality and chemical safety (THM levels) of the treated fish were taken into consideration.

6.2 MATERIALS AND METHODS

6.2.1 Process wash water

Frozen *Pangasius* fish was purchased from a Belgian supermarket and was further thawed as described in § **5.2.3** . The thawed fillets were washed in the laboratory in a dynamic system which consisted of a water bath, a sodium hypochlorite pump, and a sensor for the pH, temperature and redox potential of the wash water. The fillets were washed in chlorinated water and in tap water as the control.

The ratio of fish to water was *ca.* 1 to 10 (one fillet to 2 L of water). The fish was manually washed for 40 s in each batch. The waiting time between batches was 30 s. This waiting time was based on that observed in an earlier investigated *Pangasius* processing company in Vietnam (**Chapter 5**). Each experiment, 12 batches of fillets were washed in the same wash water. The pH of the washing water was set at pH of 6.5 (by means of 1M HCl) for every batch that was washed. The sodium hypochlorite (24.5 g/L NaOCl, Loda, Belgium) was continuously pumped to maintain the level of free chlorine in the water at 10 ppm. The concentration of free chlorine was monitored every 1 min and the flow rate of the pump was

manually adjusted where necessary. Tap water was added after every batch (i.e. one fillet) to compensate for the water lost during washing. The temperature of the washing water was maintained between 8-10°C by placing ice underneath the washing tank. Two independent repetitions (repetition 1 and 2) with chlorinated water were performed. As control experiment (non-chlorinated water), tap water was used as wash water. Before experiment performed, the tap water was contained in the clean tanks for a week storage at 2-4°C.

In this study, the effect of chlorine on decontamination of the fish surface was evaluated by means of the microbiological analysis of the fish after washing in chlorinated water. In contrast, the effect of chlorine on sensory and chemical safety of fish intended for human consumption was evaluated on the fish washed in chlorinated water after rinsing for 40 s in ordinary tap water.

6.2.2 Physico-chemical analysis

The pH, temperature and oxidation reduction potential (ORP) of the wash water were measured every 10 s using a portable HACH HQ40D digital meter connected to a data logger (Hach Lange, Germany). To avoid interference of chlorine, only the wash water of the control experiment was sampled to measure the chemical oxygen demand (COD) level by means of the small-scale sealed-tube method (LCI 400; Hach Lange, Belgium). Absorbance was measured at 249 nm with a UV visible (UV-vis) spectrophotometer (UV 1601, Shimadzu, Belgium) in quartz cuvettes with a 1-cm path length (Hellma, Belgium) after filtration through a 0.45 μm polytetrafluorethylene filter (Macherey-Nagel, Belgium). In addition, free chlorine was measured every minute and total chlorine was measured every four minutes according to the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (Eaton et al., 2005).

6.2.3 Microbiological analysis

The water samples were taken after washing batches 1, 3, 6, 9, and 12 while the fish samples were taken after washing batches 1, 5 and 11 as representing the beginning, intermediate and final moment of the washing process, respectively. For the fish samples, representative 25 g samples were aseptically transferred into sterile stomacher bags after which 225 ml of buffered peptone water (Oxoid, Hampshire, U.K.) supplemented with the sterile neutralizing agent (0.1 M $Na_2S_2O_3$) was added. The analysis of the total aerobic psychrotrophic counts,

lactic acid bacteria, *E. coli* and coliforms was performed as described in § **5.2.3.1** The media and incubation conditions for these microbial parameters are described in § **5.2.3.1** in **Chapter 5.**

For the chlorine experiment, 50 ml of water samples was aseptically taken and transferred into sterile Falcon tubes containing the sterile neutralizing agent. Due to the presence of fish matrix in the wash water, a filter membrane approach could not be executed. To lower the quantification limit for the chlorinated water samples, ten ml of non-diluted sample were distributed over 4 plates of Plate Count Agar (PCA, Oxoid, Hampshire, U.K.) and de Man Rogosa Sharpe agar (MRS, Oxoid, Hampshire, U.K); and 1 ml of non-diluted sample were spread over 4 plates of Rapid *E. coli* 2 Agar (Bio-Rad, Marnes-La-Coquette, France). For the control experiment with non-chlorinated water, serial decimal dilutions of the water samples were then prepared in physiological saline solution (1g neutralized peptone and 8.5 g NaCl per L). One ml of the tenfold diluted water samples was plated on PCA, MRS and Rapid *E. coli* 2 Agar. The incubation conditions for these plates are described in § **5.2.3.1** in **Chapter 5.**

6.2.4 Sensory evaluation

Triangle tests were used to determine if washing in chlorinated water has an influence on sensory quality of fish. After washing in chlorinated water, fillets from batch 2, 3, 4, 6, and 12 were rinsed for 40 s in ordinary tap water. The fillets washed in tap water were used as the reference. Thereafter, the fillets were cut into *ca.* 50 g pieces and randomly coded with three digit numbers for sensory evaluation of fresh fish. 12-19 panelists participated in the tests. The procedures followed were based on the ISO 4120:2004 Sensory analysis-Methodology-Triangle test (ISO, 2004) as described in § **5.2.3.3** in **Chapter 5.**

6.2.5 Disinfection by-products

The formation of halogenated by-products such as chloroform, bromoform, dichlorobromomethane, and dibromochloromethane, was determined by means of head space-gas chromatography/mass spectrophotometry as described by López-Gálvez et al. (2010a). THMs were analyzed in the water and in the treated fish sampled in the final batch (batch 12, = after 15 min. of treatment). The fish samples were rinsed in ordinary tap water for 40 s before analyzing.

6.2.6 Statistical analysis

For each experiment two independent repetitions were performed. Each repetition was analyzed in duplicate. The results of the microbiological analysis of the water and fish were expressed as log CFU/100 ml and log CFU/g, respectively. The results are reported as the mean value ± standard deviation. Cross-correlations were tested by means of the non-parametric Spearman rank order correlation coefficient (r) two tailed test ($\alpha = 0.05$) in SPSS version 20 version (IBM Inc., Chicago, Ill., USA).

6.3 RESULTS

6.3.1 Evolution of the physico-chemical properties of the wash water during processing

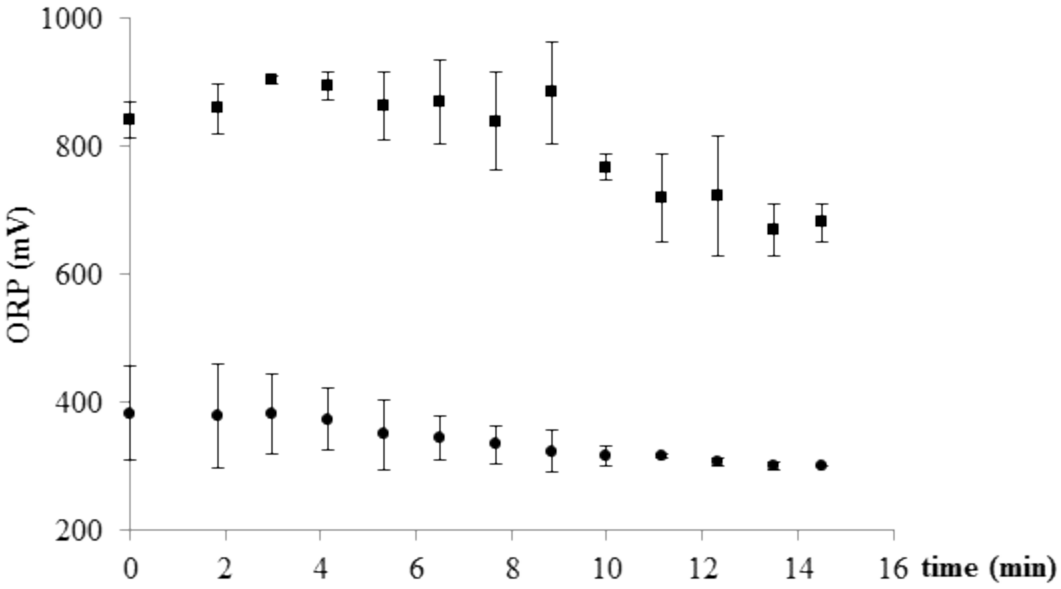


Figure 6.1 Changes in ORP (oxidation-reduction potential) of wash water during treatment with chlorinated water (■) and non-chlorinated water (●) (mean value ± standard deviation of 2 repetitions)

As can be seen in **Figure 6.1**, the initial oxidation-reduction potential (ORP) of chlorinated water was 838.6 ± 23.1 mV and remained between 860 and 880 mV through the first seven batches (up to batch 7 or 9 min of washing). Thereafter, the ORP decreased to i.e. 719.3 ± 68.5 mV (batch 9) and gradual decreased down to 681.0 ± 29.5 to batch 12 (up to 15 min of washing). It can also be seen in **Figure 6.1** that the ORP of the ordinary tap water (the control) was initially 383.6 ± 74.2 mV and gradually decreased to 300.9 ± 0.1 mV at the end of the washing process (12 batches). The chlorine concentration of ordinary tap water before

washing was lower than detection limit (<0.5 ppm). As can be seen in **Figure 6.2A**, the initial concentration of free chlorine in the wash water of the first repetition was 36.4 ppm and increased to 58.6 ppm after the first batch was washed. Thereafter, it remained 58.6 ppm up to batch 4 of washing (*ca.* 5 min of washing). Then, the free chlorine concentration declined rapidly to *ca.* 7 ppm after batch 7 was used. The initial free concentration of chlorine in the wash water used in the second repetition was much lower than that of the first repetition. As observed in the first repetition, the free chlorine concentration increased to *ca.* 30 ppm at 2.5 min; followed by a rapid decline to *ca.* 10 ppm after 6 min of washing. Subsequently, the free chlorine concentration rose up to *ca.* 20 ppm at *ca.* 8 min before decreasing to *ca.* 8 ppm as from 11 min of washing (**Figure 6.2A**). The initial total chlorine was 47.4 and 21.3 ppm in the wash water used in the first and second repetitions, respectively. In difference to the trend observed for the free chlorine, the concentration of total chlorine increased steadily throughout the washing to *ca*. 170, 340 and 480 ppm after *ca.* 5, 10 and 15 min, respectively (**Figure 6.2B**).

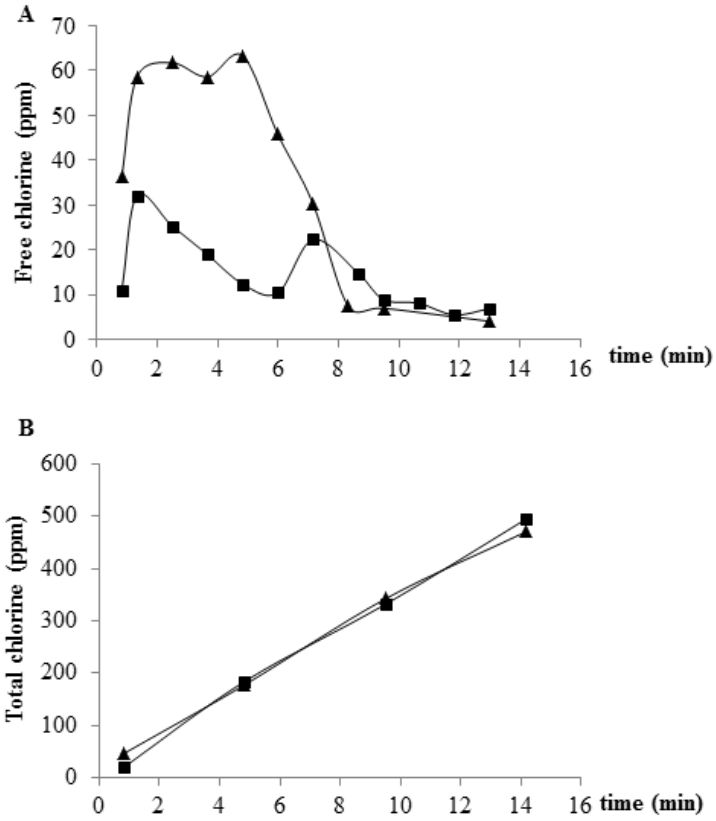


Figure 6.2 Concentration of free chlorine (A) and total chlorine (B) during treatment, 1st (▲) and 2nd repetition (■) are reported.

The pH of ordinary tap water before washing was 8.0 ± 0.2. During washing, the pH of tap water ranged from 7.3 to 7.7. The initial pH of chlorinated water was 6.5 ± 0.8 and ranged from 6.5 to 6.9 during the washing. The temperature of the different wash waters ranged from 7.2 to 8.2°C during the washing process.

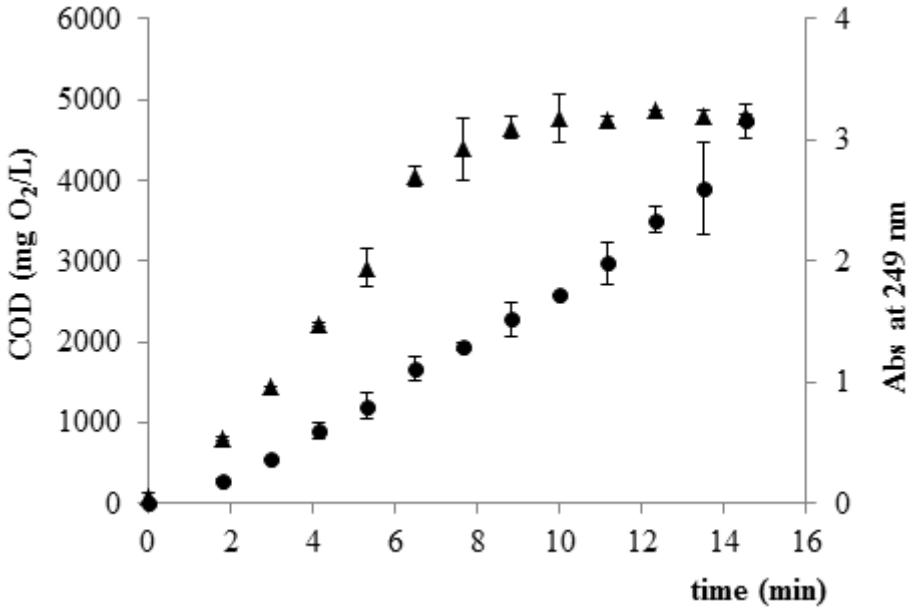


Figure 6.3 The profile of chemical oxygen demand (COD, ▲) and Absorbance (UV at 249 nm, ●) of wash water during control treatment with tap water**.**

The COD level increased gradually from 16.8 ± 3.9 (at t = 0 min) to 4735 ± 219.2 mg O_2/L (at t = 15 min, after washing of batch 12). The absorbance (at 249 nm) of tap water was initially 0.04 ± 0.03 and increased gradually during washing to a maximum value of *ca*. 3 as from *ca.* 9 min of washing onwards (**Figure 6.3**). A strong correlation was observed between the COD level and absorbance (r = 0.949 for entire washing process). Negative correlation coefficients of -0.773 and -0.764 were also determined between ORP and COD and between the ORP and absorbance, respectively. A relatively strong correlation of r = 0.859 was observed between the ORP and free chlorine.

6.3.2 Evolution of bacterial counts

The initial lactic acid bacteria (LAB), total aerobic psychrotrophic counts (TPC), *E. coli* and coliforms counts of the tap water used in this study were all below the limit of quantification for TPC and LAB (<3 log CFU/100 ml); and below limit of detection for coliforms and *E. coli* (< 2 log CFU/100 ml). The counts of LAB, TPC and coliforms in the tap water increased

from the moment fish entered the washing bath during the washing process and stayed high during the washing process. Specifically, the TPC rose sharply up to 5.6 ± 0.8 log CFU/100 ml after the first batch was washed (*ca.* 2 min). Thereafter, the TPC increased slightly to a maximum level of 6.5 ± 0.2 log CFU/100 ml after batch 9 (or 11 min). The same trend was observed for the counts of LAB and coliforms in the non-chlorinated water during the washing process. In difference, the *E. coli* counts remained below the detection limit (< 2 log CFU/100 ml) during washing in non-chlorinated tap water **(Figure 6.4)**

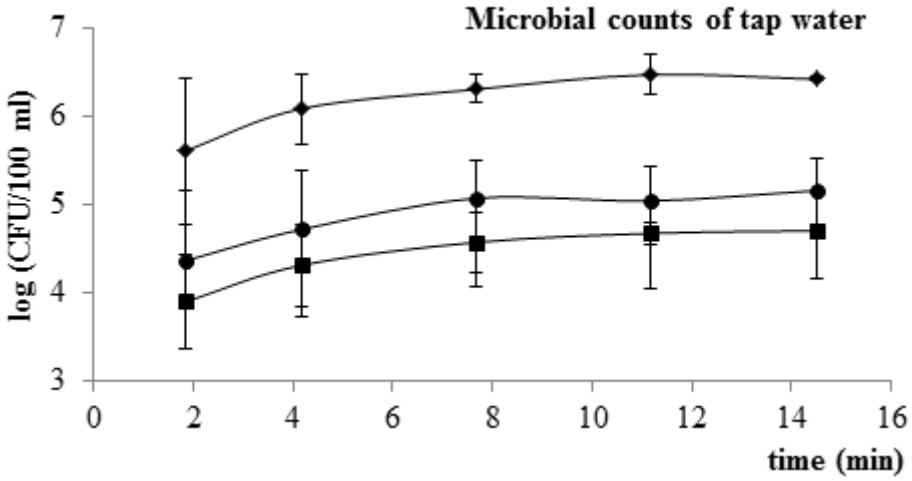


Figure 6.4 Profile of microbial counts of washing with tap water (control experiment),◆ total aerobic psychrotrophic counts (TPC), ● lactic acid bacteria (LAB), and ■ coliforms. *E. coli*: no colonies were detected in 1 ml of non-diluted wash water samples (<2 log CFU/100 ml)

In the experiment performed with chlorinated water, the initial LAB, TPC, coliforms and *E. coli* counts were all below the limit of detection (<1 log CFU/100 ml for TPC and LAB; and <2 log CFU/100 ml for coliforms and *E. coli*). A TPC of 3 log CFU/100 ml after three batches of fillets washed was seen in the chlorinated water in the first repetition. Thereafter, the TPC reduced to below the detection limit (<1 log CFU/100 ml) during the rest of the washing process. The LAB counts were 2.4 log CFU/100 ml and then decreased to below the limit of detection (<1 log CFU/100 ml) during the washing process (batch 6 onwards). Both coliforms and *E. coli* were less than the detection limit (<2 log CFU/100 ml) (**Table 6.1**). During the second repetition, the TPC of the chlorinated wash water was *ca.* 4 log CFU/100 ml after the first batch was washed and thereafter gradually decreased to 3 log CFU/100 ml after batch 9 was washed (**Table 6.1**). Thereafter, the TPC decreased to counts below the detection limit

(<1 log CFU/100 ml). The LAB, coliforms and *E. coli* counts of the chlorinated water used in the second repetition followed the same trend as that seen in the first repetition (**Table 6.1**).

Table 6.1 Microbial counts of chlorinated wash water used in the 1st and 2nd repetition

Water tested after washing batch N#	Experiment 1				Experiment 2			
	TPC*	LAB*	*E. coli***	Coliforms**	TPC	LAB	*E. coli*	Coliforms
1	3.1	2.4	<2.0[b]	<2.0[b]	3.9	2.7	<2.0[b]	<2.0[b]
3	3.0	2,1	<2.0[b]	<2.0[b]	3.7	2.7	<2.0[b]	<2.0[b]
6	<1.0[a]	<1.0[a]	<2.0[b]	<2.0[b]	3.7	<1.0[a]	<2.0[b]	<2.0[b]
9	<1.0[a]	<1.0[a]	<2.0[b]	<2.0[b]	3.1	<1.0[a]	<2.0[b]	<2.0[b]
12	<1.0[a]	<1.0[a]	<2.0[b]	<2.0[b]	<1.0[a]	<1.0[a]	<2.0[b]	<2.0[b]

*TPC and LAB: Limit of quantification (LOQ) = 2 log CFU/100 ml, limit of detection (LOD) = 1 log CFU/100 ml

***E. coli* and coliforms: Limit of quantification (LOQ) = 3 log CFU/100 ml, limit of detection (LOD) = 2 log CFU/100 ml

[a]: no colonies were detected in 10 ml of non-diluted wash water samples

[b]: no colonies were detected in 1 ml of non-diluted wash water samples

Samples of *Pangasius* fillets were taken for microbiological analysis at the beginning, middle and end of the washing process. The initial counts of TPC, LAB, coliforms and *E. coli* were 6.0 ± 0.1, 4.8 ± 0.8, 4.1 ± 0.1 and <1 log CFU/g (detection limit = 1 log CFU/g), respectively. No significant differences were found between the microbiological qualities of the fillets treated in non-chlorinated and chlorinated water. No significant differences were also found between the *Pangasius* fillets sampled at the beginning, middle and end of the washing process. The counts of TPC, LAB, coliforms and *E. coli* on the *Pangasius* fillets washed in chlorinated water were between 5.9-6.0, 4.8-4.9, 3.9-4.0 and 1.2-1.5 log CFU/g, respectively, whereas the same microbial parameters on *Pangasius* fillets washed in non-chlorinated tap water were between 5.7-6.2, 4.2-5.0, 3.9-4.4 and 1.2-1.5 log CFU/g, respectively.

6.3.3 Sensorial quality and chemical safety of **Pangasius** *fillets washed in chlorinated and non-chlorinated water*

The effect of washing the fillets with chlorinated water on the sensorial quality (color and odor) was evaluated by means of Triangle tests in which fillets washed with non-chlorinated water were designated as the reference samples. No significant differences ($p > 0.05$) were found between the sensorial qualities of the fillets washed in chlorinated and non-chlorinated water up the fourth batch (**Table 6.2**). However, a significant difference ($p < 0.05$) was found between the sensory qualities of the fillets treated in non-chlorinated and chlorinated water as from batch 6 onwards. More whitened fish and chlorine odor were noticed to differentiate between the fillets treated in chlorinated water and non-chlorinated water as from batch 6 onwards.

Table 6.2 Sensory evaluation of *Pangasius* in a consecutive washing with chlorine

Fish tested after washing in batch	Number of assessors	Number of correct responses	Required number of correct responses	Significant difference ($p < 0.05$)
2[a] (1*)	12	5	8	No**
3 (2)	13	7	8	No
4 (2)	17	9	10	No
6 (1)	12	10	9	Yes
6 (2)	19	12	11	Yes
12 (1)	12	11	8	Yes
12 (2)	13	8	8	Yes

*Number in bracket indicated the repetition (1 or 2), **indicated whether a significant difference in sensory quality between fish washed with chlorine and without chlorine. [a]sequence number of washing batch evaluated sensorially (12 batches in total in this study)

The chemical safety of the washing processes was evaluated by determining THMs production on fish and chlorinated water samples collected after the final batch (batch 12) was washed. The fillets treated in chlorinated water were rinsed in tap water for 40 s before testing the THMs amounts. The levels of THMs on the fillets washed in chlorinated water were lower than the detection limit of 4.2 µg/kg. In the chlorinated wash water itself, 8.9 ± 1.3 µg/L THMs were measured. To maintain the desired pH of 6.5, a dose of 7.3 ± 1.8 ml/L 1 M HCl was neccesary for the 12 batches. Due to the accumulation of organic matter during washing, a cumulative amount of 807.05 ± 97.4 mg/L of the sodium hypochlorite had to be used to maintain residues of free chlorine in the water (**Table 6.3**).

Table 6.3 Chlorine and HCl consumption and disinfection by-products production (detection limit of THMs is 4.2 µg/L or µg/kg) in the washing process with chlorinated water after washing 12 batches (n = 2)

Dose	Tap water	Chlorinated water[1]
Cumulative NaOCl dose; mg/L	-	807.05 ± 97.4
Cumulative HCl 1M dose; ml/L	-	7.3 ± 1.8
THMs (water); µg/L	<4.2	8.9 ± 1.3
THMs (fish); µg/kg	<4.2	<4.2

[1]Chlorinated water sampled for trihalomethanes (THMs) at the end of washing process corresponded with COD of 4447.5 ± 187.4 mg O_2/L and total chlorine of 482.9 ± 17.0 ppm in the water; (-): not determined.

6.4 DISCUSSION

Chlorine is an easy to use, reasonably priced and relatively strong disinfectant. However, chlorine rapidly decomposes in the presence of organic matter. Grace Ho et al. (2011) reported that up to 90% of residual free chlorine was lost after one batch (272.3 kg) of chopped romaine lettuce (product/chlorinated water = 1/6.5 by weight) was washed in an enclosed wash system with no replenishment of chlorine. A laboratory study on the decontamination of *Pangasius* fish by chlorinated water (product/chlorinated water = 1/10 by weight) determined that 53 to 83% of chlorine was lost after a single batch treatment (**Chapter 5**). Previously, a study carried out in a Vietnamese *Pangasius* processing company showed that high microbial counts in chlorinated wash water may result in a high risk of cross contamination for the washed products because no adjustment of pH and the chlorine concentration during washing of multiple batches was performed (**Chapter 4** and **Chapter 5**). Therefore, washing processes utilizing chlorinated water can be improved by means of maintaining a residual level of free chlorine in the wash water with a significant level of antimicrobial activity.

In this study, the residual amount of free chlorine in the wash water could not be controlled sufficiently to have at all times a desired concentration of 10 ppm. The short contact time during washing (40 s) and a short time between batches (30 s) combined with the sampling and measuring of the chlorine concentration resulted in this difficulty. Whilst the ORP of chlorinated water was positively correlated with the level of free chlorine (correlation coefficient = 0.859), it was negatively correlated with the COD levels (r = – 0.773). A recent

study on fresh cut lettuce also reported that the ORP reduces with an increase in the COD (Gómez-López et al., 2014). This study also determined that the ORP increased during treatment with chlorine while the COD levels are constant. Therefore, ORP can potentially be used as an indicator of free chlorine levels in chlorinated wash water. In this way rapid ORP measurements can be used to replace the commonly used but time consuming colorimetric methods. However, according to Gómez-López et al. (2014), ORP values cannot be used accurately to indicate the disinfection efficiency. This was a result of the observation that the inactivation of *E. coli* O157:H7 after treatments with free chlorine levels of 1 and 3 ppm was significantly different whilst the ORP values were not significantly different.

Moreover, in this study, the easier and simpler measurement of UV at 249 nm enables to estimate COD levels in the wash water due to their good correlation (r = 0.949). It is likely that the quantity of organic matter and residues of free chlorine in the wash water can be estimated more rapidly and conveniently by UV spectrophotometer and oxidant redox potential (ORP), respectively. It is suggested that the relationships among these values should be further investigated and modeled to better monitor and adapt the process. In addition, a common concern among the workers at the washing processes is that exposure to chlorine vapor may irritate the skin, eyes and respiratory tracts of humans (Abadias et al., 2008; White, 2010).

The evolution of the counts of TPC and LAB in the chlorinated water followed a similar trend in both of the independent repetitions performed. However, the microbial counts in the second repetition were higher than those in the first repetition due to the lower free chlorine concentrations in the wash water used in the second repetition. In general, the microbial counts in the chlorinated water bath decreased during the entire washing process (<1 log CFU/100 ml after washing 12 batches) due to chlorine being provided continuously. In contrast, the TPC in chlorinated water used for washing *Pangasius* products in Vietnam ranged 6.5-8.5 log CFU/100 ml (**Chapter 4**) and 5.5-6.9 log CFU/100 ml (**Chapter 5**). Although the free chlorine levels in the wash water during the last half of the washing process were only 10 to 20 ppm, *ca*. 300 to 500 ppm of total chlorine had been accumulating which could have contributed to the reduction of the bacterial counts. The large difference in the concentrations of free and total chlorine shows that a high turnover of chlorine into combined chlorine (i.e. monochloramine, NH_4Cl) can occur in the wash water (White, 2010). Combined chlorine needs a longer time or a higher concentration to inactivate bacteria than free chlorine

due to its slower disinfection action (Suslow, 2008). The antimicrobial effect of the chlorine was evident as the microbial counts in non-chlorinated wash water gradually increased during the washing process. This can be explained by a microbial proliferation in the wash water due to the large nutrients released from suspended matter (Ragaert et al., 2007). However, the counts of coliforms and *E. coli* in the non-chlorinated wash water used in this study were lower than those observed in the non-chlorinated wash water at a Vietnamese *Pangasius* processing company (**Chapter 5**). In the current study, the *E. coli* counts were below detection limit (<2 log CFU/100 ml) throughout the washing process whereas they reached a maximum of 4.6 log CFU/100 ml during washing in the non-chlorinated wash water of the Vietnamese company (**Chapter 5**). The differences observed could be a result of potentially higher *E. coli* counts on the fillets evaluated in the Vietnamese processing company which were washed after the trimming step in comparison to the *E. coli* counts that would be found on thawed frozen fillets used in this study.

E. coli counts on the *Pangasius* fillets have been found to vary greatly. For example, *E. coli* on 16 thawed frozen *Pangasius* fillets sampled in 2013 (from the same brand as those evaluated in this study) were determined to be 2.3 ± 0.7 log CFU/g (data not shown) whilst the three fillets evaluated in this study were purchased in 2014 and all had <1 log CFU/g *E. coli*. The variation observed in counts of *E. coli* on *Pangasius* products can be explained by the fact that the microbiota depends largely on the processing chain, sources of material, hygiene conditions and the performance of food safety management systems. As a result of the low *E. coli* counts on the *Pangasius* samples, it was not possible to observe a reduction in this study. This partly explained why these results were in contrast with the earlier findings observed in **Chapter 5.**

In addition, no reduction of TPC, LAB and coliform counts of the fish was seen when the fillets were washed in chlorinated water. No significant differences were also observed between the microbial quality of *Pangasius* fish processed on processing lines utilizing chlorinated and non-chlorinated water (**Chapter 2**). This highlights the fact that the purpose of using a disinfectant in the wash water should not aim only at decontamination of the fish but also disinfection of the wash water to avoid cross contamination during washing.

Discoloration (bleaching) of the surfaces of the fillets as well as a chlorine odor was a major defect in fillets washed in the final batch (batch 12). The color and odor of the fillets treated in chlorinated water till batch 4 changed to a smaller extent and did not differ significantly ($p >$

0.05) from the controls. Up to date, very few studies have reported the impacts of chlorine-based disinfection on the sensory attributes of fresh fish. A slight discoloration (melanosis) and chlorine odor were observed on shrimps whereas discoloration of skin (lighter color), chocolate color in the gills and changes in the color of the eyes occurred on whole salmon treated in a brine with 100 and 200 ppm chlorine dioxide (product/brine volume ratio = 1/4) for 5 min with continuous stirring (Kim et al., 1999a). Another study also pointed out that free chlorine levels above 200 ppm induced sensory changes in fish fillets, however, no specific sensory attributes were reported (FAO/WHO, 2008).

Besides the adverse sensory impact of chlorine, excessive chlorination can lead to greater levels of residues in the washed fillets and hence to possible health risk. THMs in the thawed frozen *Pangasius* fillets used in this study were negligible. The THMs in the fillets sampled in the last batches were lower than the detection limit (<4.2 µg/kg). The THMs generated into the wash water were far below the regulation adopted for drinking water in the European Union (EU, 1998a) and in the U.S. (USEPA, 2009). Although the formation of THMs in the water and products during the washing of vegetables has been extensively studied (Gómez-López et al., 2014; Klaiber et al., 2005; López-Gálvez et al., 2010a; Van Haute et al., 2013a); to our knowledge, no studies have yet been performed concerning the chemical safety of fish treated with chlorine.

6.5 CONCLUSION

Antimicrobial levels of free chlorine can be maintained in wash water by means of continuous dosing. This is useful to prevent cross contamination from water to the washed products. On the other hand, the automatic dosing of chlorine can be used to ensure the safety of the personnel (i.e. by avoiding or limiting the chances of inhalation and contact with the skin). Although the fillets were safe from the point of view of the levels of the THMs, the color and odor of these fillets were unacceptable after a number of batches washed in the same water. To ensure that both sensory quality and chemical safety are achieved, the wash water can only be limitedly reused.

Chapter 7 GENERAL DISCUSSION, CONCLUSIONS & PERSPECTIVES

7.1 Introduction

It has been reported that Vietnamese *Pangasius* products are a source of various nutrients (i.e. quality proteins, fatty acids, minerals, etc.) required for human health (Karl et al., 2010; Szlinder-Richert et al., 2011; Usydus et al., 2011). This has in part been responsible for its increasing appreciation and demand in the U.S. and European markets.

However, surprisingly only a few studies have so far been performed to assess the microbial quality of *Pangasius* products. These include Noor Uddin et al. (2013) who studied frozen *Pangasius* products marketed in Denmark and Noseda et al. (2012) who evaluated the shelf-life of thawed frozen *Pangasius* stored under different modified atmosphere conditions. Pathogenic bacteria including *Listeria monocytogenes* and *Salmonella*, have been reported on frozen *Pangasius* products exported to European countries (RASFF., 2013). For example, 22 cases of rejections were reported in 2009. On the other hand, the commercial aquaculture of *Pangasius* is still a young industry, which partly explains the current paucity on data concerning the origin as well as composition of the microbiota of *Pangasius* products throughout the processing chain. Moreover, although chlorine is used in the washing water in some processing plants in Vietnam and elsewhere, very little is known about the efficacy of this method for fish products. Most of the decontamination studies done to date have been performed on fresh-cut vegetable products (Allende et al., 2009; Gómez-López et al., 2014; Van Haute et al., 2013a; Van Haute et al., 2013b). This thesis attempted to address these gaps, with focus being directed towards i) determining the evolution of the microbial quality and safety of *Pangasius* products along Vietnamese processing lines (**Chapter 2** & **Chapter 4**), ii) the decontamination of *Pangasius* fillets by means of washing in chlorinated water or peracetic acid (**Chapter 5**) and iii) optimization of disinfection of the washing water by means of continuous dosing of chlorine to water used to wash several batches (**Chapter 6**).

The most important findings of this thesis are discussed below and future perspectives are briefly outlined.

7.2 Dynamics of microbial quality and safety of *Pangasius* products

Generally, the microbiological quality (contamination levels and identification of the contaminating microbiota) of *Pangasius* fillets during processing were determined to be dependent on the processing plant sampled. The microbiological quality of the *Pangasius*

fillets sampled from the small scale company evaluated in this thesis was lower than those sampled from the large scale company. The use of chlorine in the wash water did not have a positive effect on the microbial quality of the products during processing (**Chapter 2**). On the other hand, the performance of the food safety management systems (FSMS) of the food companies had an effect on the microbial safety of the produced products (**Chapter 4**). Variations were observed in the microbial quality of the *Pangasius* products on the market including the counts of *E. coli* from year to year (**Chapter 5** & **Chapter 6**). This implies that the quality of *Pangasius* products is influenced by many factors related to the entire production chain. Specific factors influencing the microbial quality of *Pangasius* fish are discussed below:

7.2.1 The quality of raw materials

Raw materials have a direct impact on the quality of the final products (Zugarramurdi et al., 2004). As an example, the initial microbiota of *Pangasius* sampled in two companies (small and large scale) evaluated in this study was variable due to differences in the sources of the fish raw materials. Although the microbial quality of the fish was not evaluated at the farms in this thesis, previous studies reported that the bacterial communities of farmed *Pangasius* differed between seasons (rainy and sunny seasons) and locations (fish farms) as they had different PCR-DGGE banding patterns (Le Nguyen et al., 2008). A high risk of bacterial (fish) diseases including *Edwardsiella ictaluri* (white spot), *Aeromonas hydrophila* (swollen head), *Pseudomonas dermoalba* (white tail) and parasitic diseases including gill fluke (Dactylogyrus), round worms (Nematoda), etc. was observed during the rainy season of June to July (Vu and Campet, 2009). The composition of the water, temperature, weather conditions and antibiotics used have also been reported to contribute to differences in the bacterial ecology of living *Pangasius* fish as well as in the end products (Orban et al., 2008; Sarter et al., 2007). During the sampling performed in this thesis, it was determined that the raw *Pangasius* material processed at the small company was sourced from a number of suppliers. As a result of frequent (recurring) financial difficulties encountered by the suppliers (or farmers) due to fluctuations in the prices of raw *Pangasius*, processors have dealt in recent years with an unsustainable supply of raw materials (Bush and Duijf, 2011). Therefore, the companies face difficulties in acquiring sufficient quantities of raw *Pangasius* with good chemical and microbiological quality from trusted suppliers for their processing. This leads to limitations in their choice of supplier(s) and consequently compromises are made with regards

to the quality of the raw *Pangasius* they use. In addition, according to VASEP (2011), about 45% of the *Pangasius* farming area (2.850 ha) in Vietnam has been certificated according to international standards of good agriculture practices such as the SQF 1000^{CM} (Safe Quality Food), GlobalGAP (Global Good Agricultural Practice) and Best Aquaculture Practices (BAP). These mainly control the residues of chemical contaminants used in intensive fish farming. These do not focus on the microbiological aspects, which may result in a potentially negative effect on the microbial quality of raw *Pangasius* materials in Vietnam. It is suggested the farmers pay attention not only on the basis of absence of chemical contaminants but also of the pathogenic microbial contaminants associated with intensive fish farming. In addition to the quality of *Pangasius* raw material, post-harvest handling of the fish from farm to factory also influences on the microbial quality and safety of the final products (Orban et al., 2008). Taking into account the factors discussed above and the fact that the studies performed in this thesis focused on two companies in Vietnam Mekong Delta, more studies have to be performed to have a broader overview of the microbiota (and variations therein) of fish farmed in different regions of the Mekong Delta. This could indicate regions or locations with a high risk of microbiological or parasitic contaminants on which corrective actions can be focused. Knowledge of the predominant microbiota present on raw *Pangasius* is also important with regards to the development of suitable microbial inspection protocols for raw materials and determination of appropriate processing for final products i.e. filleted products have a higher requirement with regards to the microbial quality of the raw materials than added value products (e.g. breaded and rolls *Pangasius*).

7.2.2 Processing

The evolution of the microbiota throughout the processing of *Pangasius* was reported in **Chapter 2**. In addition, the microbiological counts on the fish and in environmental samples were presented in **Chapter 4.** During the processing of *Pangasius* fillets intended to be marketed in frozen form, there are no real intervention steps in order to eliminate bacteria such as cooking. Therefore, the contaminating bacteria on fish products during processing can potentially occur at counts greater than the acceptable limits. As mentioned above, performance of the FSMS had an effect on the microbial quality and safety of the processed products. The FSMS implemented at the large scale company performed at a good level and this was evident in the acceptable microbial quality and safety of the fish products from this company. In general, the companies processing *Pangasius* products should focus their

attention on the core control and assurance activities in order to control microbiological risks (**Chapter 4**).

The main processing steps influencing the quality and safety of *Pangasius* products are discussed hereafter.

7.2.2.1 Filleting

The microbial counts on *Pangasius* products analysed at the filleting step were very variable (standard deviation = 1.0 log CFU/g for total aerobic counts and 1.5 log CFU/g for presumptive *Enterobacteriaceae*) (**Chapter 2**). The most prevalent species on the samples collected at the filleting step were *Aeromonas, Acinetobacter, Providencia* and *Morganella* spp. which are related to the endogenous microorganisms of the gills or intestinal tracts of farm raised freshwater fish (Austin, 2002; Hatha, 2002; Vivekanandhan et al., 2005; Yagoub, 2009). Contamination from the guts could have occurred when they were broken as a result of fast filleting. Indeed, observation of certain operators showed that gut perforations ranged between 28%-55% (**Chapter 4**). Therefore, the filleting step is a critical step with regards to (cross) contamination of microorganisms arising from the processing equipment (knives, tables, gloves, and cutting boards), the workers and the fillets. This was highlighted by the fact that species belonging to the *Enterobacteriaceae* were not only prevalent on the fish collected at the filleting step but also at those sampled at the trimming and freezing steps (**Chapter 2**). Secondly, the counts of presumptive *Enterobacteriaceae* varied greatly on final *Pangasius* products processed in Vietnam (2.3-4.3 log CFU/g) (**Chapter 2**) and on frozen *Pangasius* products marketed in Belgium (1.6-3.8 log CFU/g) (**Chapter 3**). Numerous notifications of too high *Enterobacteriaceae* counts contaminating Vietnamese *Pangasius* fillets exported to the EU market were reported, e.g. 9 cases in 2005 (RASFF, 2015). Moreover, the counts of *E. coli* ranged from <1.0 to 2.2 log CFU/g and from <1.0 to 2.4 log CFU/g on frozen *Pangasius* products processed in Vietnam (**Chapter 4**) and frozen *Pangasius* marketed in Belgium (**Chapter 5** & **Chapter 6**), respectively. Variation of these counts could result from the filleting step and hygiene procedures implemented in the companies.

In practice, *Pangasius* is filleted manually. Since the income of the workers depends on the amount of fish filleted in a month, the workers do fillet as fast as possible, without paying full attention to the quality of each fillet. On the other hand, a general characteristic of Vietnamese

companies processing *Pangasius* is the variability of workers due to a common turnover of workers (<1-5 years) (**Chapter 4**). Consequently, the workers who have the knowledge and experience to fillet *Pangasius* leave the companies at a regular rate. These factors have an impact on the performance of the filleting step with regards to the microbial quality attained after filleting.

Therefore, the filleting step should be investigated more elaborately with regards to the possible cross-contamination from the prevalent microbiota in the guts of *Pangasius* fish and the influence of manipulation at the filleting step on the microbiota of *Pangasius* products. It is also suggested to pay the workers not only depending on the quantity but also on the quality of the fish filleted.

7.2.2.2 Trimming

The main purpose of the trimming step is to get uniform and smooth fillets by removing fat, bones, skins etc. Trimming was done manually at the companies evaluated in this thesis. The microbial quality and microbial ecology on *Pangasius* products are highly dependent on the hygiene conditions during trimming. As an example, *Enterobacter* and *Wautersiella* spp. were identified on trimmed *Pangasius* sampled from the small scale company (**Chapter 2)**, reflecting the unhygienic handling and poor personal hygiene in the trimming step. These were linked to the high microbial counts observed on the *Pangasius* products sampled (**Chapter 2** and **Chapter 4**). Hygiene indicators (*S. aureus*, *E. coli*, coliforms) were also found on the environmental samples i.e. gloves, tables, knives, baskets, etc. (**Chapter 4**). Additionally, human pathogens such as *Klebsiella pneumonia* and *Shigella flexneri* were detected not only on sampled at the trimming step but also on *Pangasius* samples at the final processing step. The main route of transmission can therefore occur from the environment onto *Pangasius* as observed during the processing of catfish (Chen et al., 2010). Therefore, more attention should be paid to the preventive measures, hygienic design, sanitation program, personnel hygiene, frequency of cleaning and disinfection, etc. to avoid (cross) contamination at the manual trimming step (**Chapter 4**).

7.2.2.3 Tumbling

Although the tumbling step is optional, it was implemented in both companies sampled of this work. The fillets were treated with additives during tumbling. A few studies have reported the

possible use of polyphosphates for treating *Pangasius* fillets as high amounts of sodium (222-594 mg/100 g) (Orban et al., 2008) and reduction of thermal stability in the protein domains was observed (Karl et al., 2010). The use of polyphosphates in tumbling is mainly to increase the water retention capacity, reduce the drip loss, retard the oxidation of unsaturated fatty acids, and inhibit the growth of bacteria (Alvarez et al., 1996; Dziezak, 1990; Etemadian et al., 2011; Masniyom et al., 2005; Zaika et al., 1997). The delay of microbial growth (i.e. coliforms, total aerobic counts) has been effectively demonstrated on aquatic products treated with polyphosphates during storage (Etemadian et al., 2011; Thepnuan et al., 2008). Although the evaluation of this practice was not an objective of this thesis, it was determined in Chapter 3 that the water and salt content, pH and water activities of various frozen *Pangasius* marketed in Belgium differed significantly, indicating that some of the products could contain salts added during processing. Therefore, the use of additives during tumbling may influence the microbiota of the final frozen *Pangasius* products. Especially, the microbiota of frozen *Pangasi*us products affects directly the spoilage microbiota on thawed products which are widely marketed in Western markets as fresh products. It is necessary to investigate further the composition of additives used during tumbling and their impacts on the quality and safety in general and the microbiota of *Pangasius* products in particular.

7.2.3 Pangasius *products*

The origins as well as identity of the microbiota of *Pangasius* products were established in this thesis. An overview of diversity of the microbiota on *Pangasius* fish is summarized in **Figure 7.1**. A good relationship was established between the microbiota of *Pangasius* isolated during processing (**Chapter 2**) and the microbiota of commercial *Pangasius* (**Chapter 3**). The prevalent genera identified on *Pangasius* during processing in Vietnam were *Acinetobacter* (19.5% or 34/174 isolates), *Lactococcus* (13.8%) and *Enterococcus* (8%) (**Chapter 2**). These genera were also found prevalently on frozen *Pangasius* marketed in Belgium (**Chapter 3**). In addition, some genera belonging to *Enterobacteriaceae* e.g. *Enterobacter, Klebsiella, Wautersiella, Serratia* and *Shigella* spp. were found during processing and on final *Pangasius* fish products (**Chapter 2**). The origin of these genera can be in part explained by the high contamination with *Enterobacteriaceae* from the environments by means of the MAS (Microbial Assessment Scheme) performed in **Chapter 4**. The microbial safety of the final *Pangasius* products was reflected through the performance of food safety management systems (FSMS) currently applied at these companies. It is clear that

the food safety output can be greatly improved by means of implementing advanced core control and assurance activities (Jacxsens et al., 2009b; Jacxsens et al., 2010). As an example, the low microbial food safety risk of *Pangasius* products at the large company were a result of more advanced control activities (i.e. sanitation programs, personal hygiene, etc.) and mature assurance activities (i.e. validation and verification of preventive measures) (**Chapter 4**). The results obtained show that the microbial quality and safety of Vietnamese *Pangasius* products processed in the large company are quite good based on official standards and guidelines whereas more attention should be focused on the products processed in the small company due to the occurrence of *Listeria monocytogenes* and *Shigella flexneri* (**Chapter 2** & **Chapter 4**). In addition, the MAS developed by Jacxsens et al. (2009b) can give insight into the overall microbiological contamination (on fish, water, food contact surfaces and hands) from beginning until the end of processing. This assessment scheme is necessary to implement routinely in *Pangasius* processing companies in order to validate the food safety management system in place and to avoid economic losses due to microbial food safety–related product rejections summarized in **Chapter 1.**

On the other hand, Noseda et al. (2012) found that *Pseudomonas* and *Serratia* spp. were the dominant spoilage microorganisms of thawed frozen *Pangasius* products from Vietnam stored in air or modified atmosphere package (MAP) conditions (**Figure 7.1**). These genera were also identified on *Pangasius* during processing (**Chapter 2**) and on frozen *Pangasius* marketed in Belgium (**Chapter 3**). Therefore, these results explain the origin of the microbiota of processed *Pangasius* products. Despite both of them being dominant on *Pangasius*, their contribution to the spoilage of *Pangasius* still has to be determined. If species belonging to these genera have the capacity to cause product spoilage they could be used as parameters to evaluate or indicate the microbiological quality of the product. Additionally, decontamination of fresh *Pangasius* fillets should be evaluated based on these species if they are potential spoilage organisms; subsequently, the further study aims to extend shelf life of *Pangasius* stored under fresh (for Vietnamese markets) and thawed forms (for international markets).

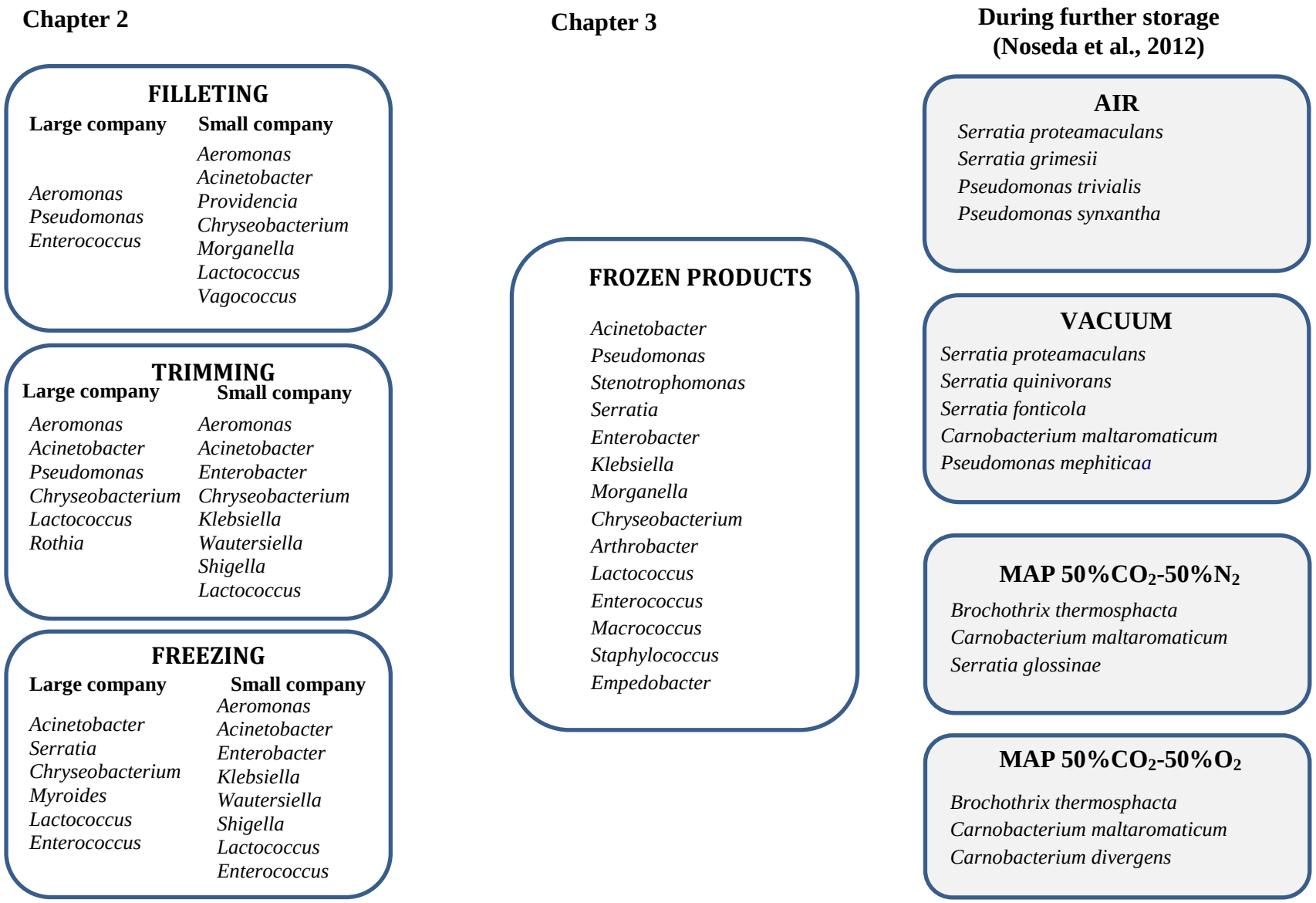


Figure 7.1 Overview of microbiota identified on *Pangasius* fish by 16S rRNA gene sequencing from the present study and previous study, large company with non-chlorine and small company with chlorine – based process.

7.2.4 Decontamination currently employed in Vietnam and its optimization

7.2.4.1 Washing performed in Vietnam

The use of chlorine as a disinfectant in the wash water was determined to have no impact on the microbial counts of the *Pangasius* fillets at a large scale processing company in Vietnam (**Chapter 2**). The counts of spoilage and pathogenic microorganisms on *Pangasius* products were influenced by several other factors including the quality of materials, (good) manufacturing practices and the performance of the FSMS (**Chapter 2** & **Chapter 4**). In addition, the processing line at the large scale company which utilized non-chlorinated water for washing of the fillets had a better food safety output than that of the small company which utilized chlorinated water for washing (**Chapter 4**). The possible reasons for the lack of effect of the chlorine in reducing the microbial loads of the *Pangasius* fillets included a lack of control of the concentration of chlorine and pH of the washing water during processing and build-up of organic matter in the chlorinated water during the washing of successive batches (**Chapter 4** & **Chapter 5**). The reuse of non-chlorinated and chlorinated wash water resulted in the build-up of the total psychrotrophic counts (TPC) in the washing water used at the small scale company in Vietnam (**Figure 7.2**). The built up of microbial loads in the washing water can cause further contamination to the washed products (Allende et al., 2009). Therefore, to improve the quality of the washing water as well as the washing process itself, some important points to be considered are listed below.

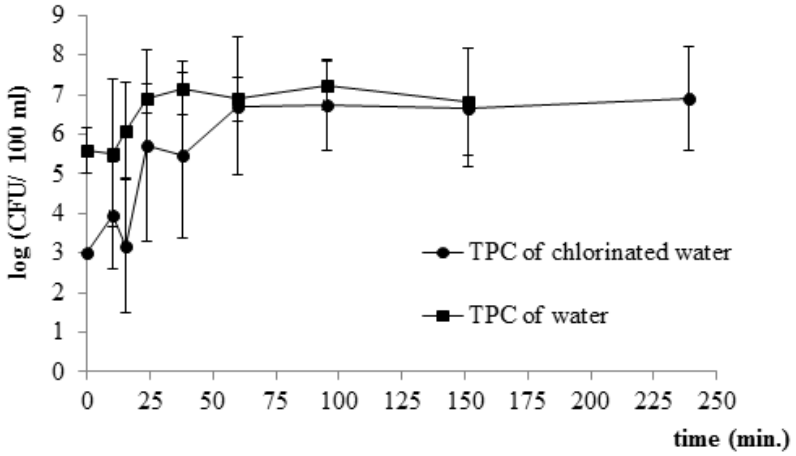


Figure 7.2 The total psychrotrophic counts (TPC) in the wash water sampled at a Vietnamese *Pangasius* processing company

7.2.4.2 Improvement of the washing process

Based on the evaluation of the actual washing process implemented in a Vietnamese company, the washing process was also simulated on a laboratory scale (**Chapter 5**).

An example of washing **a batch** is as follows:

Ratio fish/water:	1/10
pH of wash water:	6.5
Temperature:	8 – 10 ^{o}C
Initial concentration of chlorine:	44.7 ± 3.4 ppm

The free chlorine levels had decreased by 73.3 ± 20.7% after 40 s of washing, during which time the organic matter had accumulated from 16.5 ± 3.5 to 238 ± 66.3 mg O_2/L. In addition, the TPC of wash water were 3.9 ± 0.8 log CFU/100 ml (in chlorine) and 5.4 ± 0.1 log CFU/100 ml (in tap water as control) after 40 s (**Chapter 5**). As a result of rapid degradation of chlorine observed for one batch washing, it is clear that the compensation for the reacted free chlorine is necessary for the company where they applied this wash water for washing multiple batches (**Chapter 6**).

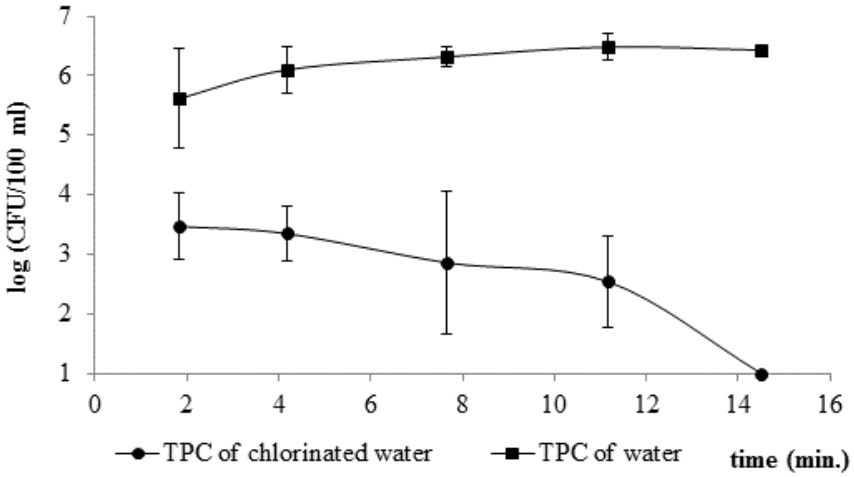


Figure 7.3 The total psychrotrophic counts (TPC) in the wash water with chlorine continuously dosed performed at the laboratory scale for washing (12 batches), limit of detection = 1 log CFU/100 ml.

The TPC counts in the wash water during the successive washing of 12 batches of *Pangasius* fillets were reduced down to the detection limit (1 log CFU/100 ml) (**Figure 7.3**). As a result of the low microbial counts of the wash water, washing with enough chlorine in the wash water is essential to prevent further (cross) contamination (Casani et al., 2005; Van Haute et al., 2013a). In addition, the company needs to systematically establish the specific limit/guidelines of microbial

levels for all washing steps (i.e. bleeding, washing in water and washing in chlorine). These limits can improve the microbial safety of products taking into consideration the high risk of cross contamination from the wash water to the *Pangasius* products observed in **Chapter 4**. Especially, no real intervention steps to eliminate bacteria (i.e. heating) are applied for frozen *Pangasius* products, hence the chemical intervention of washing fish in chlorine can prevent cross contamination and can further contribute to improve the FSMS (Luning et al., 2008).

However, the efficacy of chlorine is limited for decontaminating *Pangasius* fish washed during processing (**Chapter 2** & **Chapter 4**), in a single batch (**Chapter 5**) or in dynamic (continuous multi-batch) system (**Chapter 6**). Chlorine is much more effective for inactivation of microorganisms in wash water itself, thus for improvement of the microbial quality of the wash water, than for reduction of these microorganisms on products such as fruits and vegetables (Gil et al., 2009; Sapers, 2001) or *Pangasius* as investigated in this thesis. To improve the decontamination of *Pangasius*, it is necessary to further investigate the attachment of bacteria on *Pangasius* fish and the susceptibility of these bacteria for disinfectants because both factors influence the efficacy of the decontamination of products (Takeuchi and Frank, 2001; Van Haute et al., 2013d).

In the investigated dynamic system, the amounts and the cost of water can be reduced (Luo, 2007) because the wash water could be reused for up to 12 batches based on the chemical safety of both products and the wash water (**Chapter 6**). However, the high concentration of chlorine negatively affected the sensory properties of the products (Arbor, 2008). The sensory properties of *Pangasius* fillets washed in the final batch were discolored (bleaching) and had a noticeable chlorine odor (**Chapter 6**). To optimize the wash process by means of a dynamic chlorine dosing system, the safety of the by-products, sensorial quality of products after treatment in addition to the basic parameters (i.e. concentration, contact time, pH, organic matter, ratio product/water etc.) must be taken into account. Furthermore, some specific points are recommended for optimizing the washing process:

- Chlorine and organic matter in the wash water can be estimated faster and more convenient than N,N-diethyl-p-phenylenediamine (DPD) colorimetric and the small-scale sealed-tube method by means of ORP (oxidation reduction potential) and UV absorbance, respectively,

because of their good correlation as seen in **Chapter 6**. The free chlorine in water can be determined by using a calibrated ORP (Suslow, 2001). However, the relationships of these values should be further investigated and modeled to get more accurate estimation. From this, the washing based on an on-line monitoring and controlling system (for direct control of the feed pumps of chlorine) may be optimized and applied.

- In general, numerous studies on decontamination of artificially inoculated vegetables by sanitizers have been reported in **Chapter 1**. Only a few studies have reported the efficacy of these processes; however, up to date no inoculation studies on *Pangasius* have been reported. The natural background microbiota of thawed frozen *Pangasius* performed in this thesis (**Chapter 5** & **Chapter 6**) had low numbers of bacteria so no conclusions could be made on the effect of chlorine on the target spoilage bacteria (e.g. lactic acid bacteria) or pathogenic bacteria (e.g. *Shigella* or other *Enterobacteriaceae*) obtained in **Chapter 2** and **Chapter 4**. This should be studied further by means of inoculating these target bacteria onto the surfaces of the fish (as the worst case) and evaluating thereafter the decontamination efficacy of chlorine on these fish.
- To limit the accumulation of organic matter as well as chlorine-consumed, *Pangasius* fish should be pre-washed (or sprayed) in tap water to remove surface located organic matter prior to treatment in chlorinated water. On the other hand, the fish treated in chlorinated water should be further rinsed (by spraying) by tap water. As a result of this, the dose of chlorine used can be reduced and the risk of formation of harmful trihalomethanes can be limited.
- The antimicrobial activity of chlorine also depends on the pH of the water. Therefore, the companies should adjust the pH of the water to the optimal antimicrobial range of 6.5 to 7.5. This will also prevent the formation of chlorine gas which can affect the health of the workers and corrode the equipment (especially when the process operates at low pH of 5).

7.2.4.3 Alternative disinfectant

Prolonged exposure to vapor chlorine can ultimately affect the heath safety of the workers in the washing areas. Additionally, the risk of formation of by-products i.e. trihalomethanes by over-chlorination in the wash water containing high amounts of organic matter can also result in a negative effect to humans. Therefore, a good alternative to chlorine in wash water used for several batches may be peracetic acid as the loss of peracetic acid due to the presence of organic matter is much lower than that of chlorine during washing (**Chapter 5**). It is also active over a wider pH range and produce far less toxic by-products. In addition, some countries in the EU including Belgium, Germany, Switzerland, Denmark and the Netherlands are not allowing the products

being washed in chlorinated water (EU, 2004b). Despite the the high cost, the alternative of peracetic acid could be a good option for processors.

7.3 Concluding remarks

Pangasius production has boomed rapidly in Vietnam with the quantity of exported *Pangasius* fillets increasing *ca*. 1000 times from 7,000 in 1997 to 726,000 tons in 2011 (VASEP, 2014). However, the sustainable development of *Pangasius* aquaculture is still a big challenge (Phan et al., 2009; Sinh, 2007; Van Sanh and Van Binh, 2013) due to the existence of many problems from the production to the processing phase (i.e. interaction between the *Pangasius* culture and the environment, farming technologies, supply chain development, quality control, etc.) (Van Sanh and Van Binh, 2013). However, the big gap concerning how to guarantee the microbial quality and safety of *Pangasius* catch to consumer is still not filled yet up to date. Therefore, the results obtained from this study fill part of the this gap and can therefore have a significant impact on the microbial quality and safety of Vietnamese frozen *Pangasius* products destined for both local and international trade. To obtain this impact however, several implementation steps at different levels should be taken. Firstly, the GlobalGAP standard currently covers good aquaculture practices on the farm to control especially chemical contaminants during the farming of *Pangasius*. It is recommended that GlobalGAP should be expanded to cover biological hazards as well such as bacterial pathogens, parasitic and virus contaminants. As a result of this, the control of the microbial and chemical hazards of raw *Pangasius* can have a positive impact on the final products. Secondly at the processing level, microbial contaminants can initially be spread as from the filleting step onwards. Hence, the correct application of GMP (Good Manufacturing Practices) are considered as a crucial strategy to avoid (cross) contamination. Thirdly, chlorine can be a useful disinfectant to use in the washing steps for the purpose of avoiding (cross) contamination if correctly applied. In addition, alternative disinfectants (peracetic acid, ozone, and other organic acids) can also find its application at the industrial level. In-depth understanding and proper use of these disinfection agents is important for producers; therefore, validation of the efficacy in the washing steps is highly recommended. (Non) use of these disinfection agents for decontamination can be optional; however, good performance of food safety management greatly improves the food safety and quality output. To conclude, these strategies examined for *Pangasius* can also be implemented by companies processing other fishery products (i.e. catfish, tilapia, etc.) and seafood (shrimp, squid, etc.) in South-East Asian countries.

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APPENDIXES

Appendixes for Chapter 4

Annex 4.1 Detailed results of the MAS of *Pangasius* processing plant evaluated in the large scale plant

Number of sampling locations (SL)	N=135	Quality indicators						Pathogenic microorganisms		
		TPC*	TMC*	*E. coli*	Enterobac -teriaceae	*S. aureus*	LAB*	*L.monocytogenes*	*Vibrio* spp.	*V. cholerae*
1	3x3[1]	2.9-4.0[2]	5.3-6.4	<1.0-2.1	-	-	-	A[3]	-	-
2	3x3	3.3-4.1	3.6-5.1	<1.0-1.7	<1.0-1.6	<1.0 -1.5	<1.0-2.3	A	<1.0-1.5	A
3	3x3	2.9-4.8	-	<1.0	-	-	-	A	-	-
4	3x3	<2.0-4.4	-	<1.0	-	-	-	A	-	-
5	3x3	2.3-4.1	3.4-5.3	<1.0-1.7	<1.0-2.6	<1.0-3.3	<1.0-3.0	A	≤1.0	A
6	3x3	<2.0-2.8	3.1-3.8	<1.0	-	-	-	A	-	-
7	3x3	2.3-4.3	3.2-4.3	<1.0-1.3	<1.0-1.8	<1.0-2.7	<1.0-3.0	A	<1	A
8	3x3	2.0-4.3	-	<1.0	-	-	-	A	-	-
9	3x3	2.0-3.5	-	<1.0	-	-	-	A	-	-
10	3x3	2.0-3.4	3.0-4.1	<1.0	<1.0-1.8	<1.0-2.3	<1.0	A	<1	A
11	3x3	<2.0-4.4	<1.0-3.4	≤1.0	-	-	-	A		
12	3x3	2.0-3.3	2.9-3.9	≤1.0	<1.0-1.3	<1.0-2.1	<1.0	A		
13	3x3	<2.0-3.4	-	<1.0	-	-	-	A		
14	3x3	<2.0-3.4	-	<1.0	-	-	-	A		
15	3x3	2.5-3.6	3.6-4.2	≤1.0	≤1.0	<1.0	<1.0-2.3	A		
FS level		2	2	3	3	1	1	3	2	3

Annex 4.2 Detailed results of the MAS of *Pangasius* processing plant evaluated in the small scale plant

Number of sampling locations (SL)	N=144	Quality indicators				Pathogenic microorganisms		
		TPC*	*E. coli*	Coliforms	*S. aureus*	*L. monocytogenes*	*Salmonella*	*V. cholerae*
1	3x3[1]	4.4-5.9[2]	<1.0-1.7	1.6-3.3	-	A[3]	A	A
2	3x3	3.7-6.1	<1.0-1.8	1.0-3.1	<1.0-2.6	A	A	P (1/9)
3	3x3	4.2-7.0	1.7-4.2	2.9-5.4	<1.0-2.1	A	A	P (2/6)
4	3x3	4.6-7.5	<1.0-3.4	2.8-4.5	-	A	A	-
5	3x3	4.3-6.2	<1.0-2.3	1.7-4.3	<1.0-2.7	A	A	P(1/9)
6	3x3	5.1-6.6	1.7-3.7	4.1-5.7	<1.0-1.7	A	A	A
7	3x3	5.5-7.4	1.3-3.9	4.0-4.9	-	A	A	-
8	3x3	4.0-6.4	<1.0-2.6	2.3-3.6	-	A	A	P (1/9)
9	3x3	5.3-6.7	1.8-3.1	3.2-4.7	<1.0-3.0	A	A	P (2/9)
10	3x3	4.5-6.5	<1.0-2.6	2.3-3.5	-	A	A	A
11	3x3	5.5-6.5	2.2-3.5	3.2-4.7	<1.0-2.5	A	A	A
12	3x3	3.6-5.8	<1.0-2.9	2.2-3.6	<1.0-3.1	A	A	P (2/6)
13	3x3	4.6-7.0	1.2-3.4	3.1-4.8	<1.0-2.7	A	A	A
14	3x3	4.1-6.2	<1.0-1.1	2.0-3.4	<1.0-1.9	A	A	P(2/6)
15	3x3	3.5-6.6	<1.0	1.6-3.2	-	A	A	-
16	3x3	4.6-5.9	<1.0-2.2	2.8-4.5	<1.0-3.0	P (1^4/9)	A	P(4/9)
FS level		1	1	1	1	1	3	1

[1]3 subsequent visits x 3 independent sampling days

[2] Microbiological count range for fish (log CFU/g), hands and surfaces (log CFU/100 cm^2) and water (log CFU/ml). Value with < symbol are below quantification limit

[3]A: absence and P: presence in 25g or 100 cm^2 or 1 ml.

[4] The number of positive samples for a particular pathogen on the total number of samples.

* TPC: Total psychrotrophic plate count; TMC: Total mesophilic plate count; LAB: lactic acid bacteria; (-): not determined

Appendixes for Chapter 5

Annex 5.1 Statistical table to evaluate Triangle test. Entries are the minimum number of correct responses required for significance at the stated significance level % (i.e. column) for the coresponding number of panelist "n" (i.e. row).

Significance level (%)					Significance level (%)				
n	**10**	**5**	**1**	**0.1**	**n**	**10**	**5**	**1**	**0.1**
3	3	3	-	-	**26**	13	14	15	17
4	4	4	-	-	**27**	13	14	16	18
5	4	4	5	-	**28**	14	15	16	18
					29	14	15	17	19
					30	14	15	17	19
6	5	5	6	-	**31**	15	16	18	20
7	5	5	6	7	**32**	15	16	18	20
8	5	6	7	8	**33**	15	17	18	21
9	6	6	7	8	**34**	16	17	19	21
10	6	7	8	9	**35**	16	17	19	22
11	7	7	8	10	**36**	17	18	20	22
12	7	8	9	10	**42**	19	20	22	25
13	8	8	9	11	**48**	21	22	25	27
14	8	9	10	11	**54**	23	25	27	30
15	8	9	10	12	**60**	26	27	30	33
16	9	9	11	12	**66**	28	29	32	35
17	9	10	11	13	**72**	30	32	34	38
18	10	10	12	13	**78**	32	34	37	40
19	10	11	12	14	**84**	35	36	39	43
20	10	11	13	14	**90**	37	38	42	45
					96	39	41	44	48
21	11	12	13	15					
22	11	12	14	15					

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SUMMARY

The thesis investigated how the microbial quality of *Pangasius* is influenced during processing. In **Chapter 1**, a literature review was summarizing various aspects of the processing of *Pangasius* products. The overview of the growing global socio-economic importance of Vietnamese *Pangasius* products was described. *Pangasius* products have been reported to be good sources of valuable nutrients e.g. polyunsaturated fatty acids, minerals, (non) essential amino acids, etc. The studies performed to date on the microbial quality and safety of freshwater fish and *Pangasius* in particular were summarized in this chapter. It appears that very little information is available about the microbiota of *Pangasius* fillets during processing as well as the microbiological quality of *Pangasius* products themselves. Knowledge of the evolution of the microbiological quality during processing is essential to identify and apply corrective actions at processing steps which are critical for contamination of the products. Lastly, it was evident in literature that whilst the decontamination of fish with chlorine is practiced in some processing companies only a few studies have reported the efficacy of these processes. The focus of most of studies on disinfection has been on fresh-cut vegetables. An overview of the applications of chlorine and peracetic acid (as an alternative disinfectant) is also presented in the literature review together with some examples of their application on vegetables.

In **Chapter 2**, culture-dependent methods were used in the first part of the study to determine the evolution of the microbiota of *Pangasius* fillets throughout the processing lines at two companies (large and small scale) located in the Mekong Delta of Vietnam. The large scale company employed the use of both chlorinated and non-chlorinated washing water (on separate processing lines), whilst the small scale company only used chlorinated wash water. The microbiological counts during processing in the large company were observed not to be significantly different ($p > 0.05$) between the fillets processed on the lines utilizing chlorinated and non-chlorinated wash water. Surprisingly, fillets washed in chlorinated water from the small scale company were revealed to have significantly higher microbial counts ($p < 0.05$) than the fillets washed with potable water at the large scale company. It was determined that temperature abuse during processing in the small scale company partly explained the high microbial counts observed on their fillets. The second part of Chapter 2

identified to the species level the spoilage related microbiota of *Pangasius* fillets by means of 16S rRNA gene sequencing. On a total of 174 isolates, 20 genera and 38 species were identified along the processing chains. The microbiota related to spoilage on both processing lines at the large company was more diverse than those on the processing line of the small scale company. In general, the genera *Aeromonas, Acinetobacter, Lactococcus* and *Enterococcus* were prevalent at various processing steps on all of the processing lines evaluated. *Serratia* spp. was only observed on fillets sampled on both processing lines of the large company whereas a diverse range of isolates belonging to *Enterobacteriaceae* such as *Providencia, Shigella, Klebsiella, Enterobacter* and *Wautersiella* were isolated from fillets sampled from the small scale company. Therefore, the results obtained reflect a potential hazard with regards to the microbiological safety of the *Pangasius* products produced at the small scale factory that was evaluated in this thesis.

In **Chapter 3,** the microbiota of frozen Vietnamese *Pangasius* products marketed in Belgium was determined. The results showed that the total psychrotrophic and mesophilic aerobic counts of the *Pangasius* products evaluated ranged from 3.8-5.2 log CFU/g and 3.8-4.8 log CFU/g, respectively. Lactic acid bacteria counts varied from 2.2 to 4.1 log CFU/g while the counts of presumptive *Enterobacteriaceae* ranged from 1.6 to 3.8 log CFU/g. Fourteen different genera and 18 different species were identified by means of 16S rRNA gene sequencing. The most prevalent genera were *Lactococcus* (31.2% of the isolates), *Staphylococcus* (11.7%), *Serratia* (10.4%), *Acinetobacter* (9.1%), *Enterococcus* (7.8%) and *Pseudomonas* spp. (6.5%). The overview of physico-chemical properties of the *Pangasius* products were also discussed in this chapter.

In **Chapter 4**, a study was conducted to evaluate the performance of the food safety management systems (FSMS) applied at the small and large companies. This was performed by means of a FSMS self-assessment tool combined with a microbial assessment scheme (MAS). The MAS was applied to the processing line at the small scale company which utilized chlorinated water and on the line using non-chlorinated washing water at the large scale company. The results showed that the microbial safety and quality of products sampled in the small company was not guaranteed as the contamination levels remained high throughout processing. *Escherichia coli*, *Staphylococcus aureus* and *Vibrio cholerae* were present on the hands of food operators, especially those in the packaging area. The presence of *Listeria monocytogenes* (1 positive out of 9 samples) on the final products was likely a

result of inadequate hygiene practice. On the contrary, low levels of total psychrotrophic bacteria (*ca.* 3 log CFU/g) and *E. coli* (below quantification limit) were found on the final products sampled from the large scale company. In addition, *L. monocytogenes* and *V. cholerae* were absent in all the samples analysed. Also discussed in this chapter are the results of a self-assessment questionnaire, which provides insight into the performance of the food safety management system currently implemented at these companies. The problems of control activities and assurance activities resulted in the high safety risk of *Pangasius* products observed in the small size company.

In the first part of **Chapter 5**, a preliminary evaluation of the decontamination efficacy of washing *Pangasius* in chlorinated water was performed at the same **small company**. As chlorine was only added at the beginning of the processing of a washing bath and was used continuously without renewal for 239 min, the total psychrotrophic counts, *E. coli* and coliforms on the *Pangasius* fillets did not reduce significantly ($p > 0.05$) after washing. This could be explained by the rapid accumulation of organic matter which resulted in a decrease in the level of free chlorine from 34.4 ± 2.9 ppm to 7.8 ± 3.6 ppm after 24 min of processing. In addition, the microbiological counts in the wash water increased to 5.7 (total psychrotrophic counts), 3.9 (coliforms) and 3.0 (*E. coli*) log CFU/100 ml after 24 min. of washing. The second part of Chapter 5 evaluated the disinfection efficacy of chlorine and peracetic acid (PAA) on both the processing water and *Pangasius* fillets on **a laboratory scale**. Washing with chlorine and PAA wash water resulted in a reduction of *E. coli* counts on *Pangasius* fish which ranged from 0-1.0 and 0.4-1.4 log CFU/g, respectively, while smaller reductions of total psychrotrophic counts, lactic acid bacteria and coliforms on *Pangasius* fish were observed. However, in comparison to PAA, chlorine was lost rapidly from the wash water. As an example, 53-83% of chlorine and only 15-17% of PAA was lost after washing for 40 s (chemical oxygen demand (COD) = 238.2 ± 66.3 mg O_2/L). Therefore peracetic acid can be an alternative sanitizer, especially in companies were the wash water is recycled and used to wash several batches. However, its higher cost will have to be taken into account. Where (cheaper) chlorine is used, the processors have to pay close attention to the residual chlorine level, pH and COD level during treatment for optimal efficacy.

Based on a screening performed in Chapter 5, **Chapter 6** evaluated the continuous dosing of chlorine in the wash water on the decontamination of the fish, the evolution of the microbial load (quality) of the wash water and the sensory and chemical safety of the washed fillets. In

comparison to the control (where non-chlorinated wash water was used), a high reduction in the microbial counts of the wash water was observed when chlorine was continuously dosed into the wash water. As an example, the counts of psychrotrophic bacteria in the wash water continuously dosed with chlorine were 2-4 log CFU/100 ml lower than those in the non-chlorinated wash water after 12 batches of filets had been washed. After the final batch of fillets were washed (batch 12), the total chlorine and organic matter in the water had accumulated up to 482.9 ± 17.0 ppm and 4447.5 ± 187.4 mg O_2/L, respectively. However, only 8.9 ± 1.3 µg/L of trihalomethanes were formed in the chlorinated wash water whereas no trihalomethanes were detected in the washed *Pangasius* fillets after rinsing. It was concluded that the *Pangasius* fillets washed in continuously chlorinated water were safe for human consumption even after 12 batches had been washed. However, the organoleptic properties of *Pangasius* fillets washed in the final batch were unacceptable due to discoloration (bleaching) and chlorine odor. Therefore, according to the sensorial data, the number of batches washed with continuously chlorinated water should be limited to four batches. It was also determined that the quantity of organic matter and residues of free chlorine in the wash water can be estimated more rapidly and conveniently by measuring UV absorbance (249 nm) and oxidation reduction potential (ORP), respectively. The continuous dosing system appears to have a great potential for application in the washing process for the fish products where several batches are washed in one water bath.

SAMENVATTING

Deze thesis onderzoekt hoe de microbiologische kwaliteit van *Pangasius* beïnvloed wordt tijdens de verwerking. In **hoofdstuk 1** vat een literatuuronderzoek de verschillende aspecten van de verwerking van *Pangasius* producten samen. Het groeiende globale socio-economische belang van Vietnamese *Pangasius* producten wordt beschreven. *Pangasius* producten zouden een goed bron zijn van waardevolle ingrediënten, o.a. polyonverzadigde vetzuren, mineralen, (niet) essentiële aminozuren, enz.. De onderzoeken die tot nu toe uitgevoerd zijn rond de microbiologische kwaliteit en veiligheid van zoetwater vis en specifiek *Pangasius* worden samengevat in dit hoofdstuk. Tot op heden is er nog maar weinig informatie voorhanden over de microbiota van *Pangasius* filets gedurende de verwerking of over de microbiologische kwaliteit van *Pangasius* producten zelf. Kennis over de evolutie van de microbiologische kwaliteit gedurende de verwerking is nochtans essentieel om de kritische stappen, leidend tot contaminatie van de producten, te identificeren en de juiste corrigerende maatregelen te treffen. Tenslotte, hoewel in literatuur duidelijk naar voren komt dat de decontaminatie van vis met chloor uitgevoerd wordt in sommige verwerkingsbedrijven, wordt de efficiëntie van dit proces maar zelden gerapporteerd. De focus van de meeste van deze studies rond desinfectie lagen op vers versneden groenten. Een overzicht van het gebruik van chloor en perazijnzuur (als een alternatief desinfectans) wordt ook weergeven in het literatuuronderzoek, naast een aantal voorbeelden van hun gebruik bij groenten.

Hoofdstuk 2 bestaat uit twee deelstudies. In het eerste deel van de studie werden cultuurafhankelijke methodes gebruikt om na te gaan hoe de samenstelling van de microbiota op *Pangasius* filets veranderen tijdens de productie van de filets. Er werden stalen genomen aan de productielijnen van twee bedrijven (een groot- en een kleinschalig bedrijf), gesitueerd in de Mekong Delta van Vietnam. Het grootschalig bedrijf had productielijnen waar gechloreerd water werd gebruikt en lijnen waar niet gechloreerd water werd gebruikt, terwijl bij het kleinschalig bedrijf enkel gechloreerd water werd gebruikt. De microbiologische tellingen van de filets die geproduceerd werden op de productielijnen met gechloreerd en niet-gechloreerd water waren niet significant verschillend van elkaar ($p > 0,05$). Verder was het verrassend dat de filets die geproduceerd werden in het kleinschalig bedrijf en gewassen werden met gechloreerd water, significant hogere microbiologische tellingen hadden dan de

filets die met drinkbaar water gewassen werden in het grootschalig bedrijf ($p < 0,05$). Temperatuurmisbruik tijdens het productieproces kon de hogere tellingen in het kleinschalig bedrijf gedeeltelijk verklaren.

In het tweede deel van de studie werden de bederf gerelateerde microbiota van *Pangasius* filets geïdentificeerd top op soortniveau met behulp van 16S rRNA gen sequenering. Een totaal van 174 isolaten, 20 genera en 38 soorten konden worden geïdentificeerd gedurende het productieproces. De bederforganismen die geïsoleerd werden bij de twee productielijnen van het grootschalig bedrijf waren meer divers dan deze geïsoleerd van de productielijn van het kleinschalige bedrijf. In het algemeen kwamen de genera *Aeromonas, Acinetobacter, Lactococcus* en *Enterococcus* voor tijdens verschillende productiestappen op alle onderzochte productielijnen. *Serratia* spp. werd alleen waargenomen op filets die afkomstig waren van het grootschalige bedrijf (beide productielijnen) terwijl een diverse range isolaten die behoorden tot de *Enterobacteriaceae* zoals *Providencia, Shigella, Klebsiella, Enterobacter* en *Wautersiella* werden geïsoleerd van filets die werden gesampled in het kleinschalige bedrijf. Daarom lijkt het erop dat er een potentieel gevaar is op vlak van de microbiologische veiligheid van *Pangasius* producten die geproduceerd werden in het kleinschalig bedrijf dat in deze studie werd geëvalueerd.

In **Hoofdstuk 3** werd de microbiota van bevroren Vietnamese *Pangasius* producten die in België werden vermarkt, onderzocht. De resultaten tonen aan dat de totale psychrotrofe en mesofiele aerobe tellingen van de *Pangasius* producten varieerden tussen 3.8-5.2 log KVE/g en 3.8-4.8 log KVE/g, respectievelijk. De melkzuur aantallen varieerden van 2.2 tot 4.1 log KVE/g terwijl het aantal vermoedelijke *Enterobacteriaceae* tussen 1.6 en 3.8 log KVE/g lag. Er werden 14 verschillende genera en 18 verschillende soorten geïdentificeerd op basis van 16S rRNA gen sequenering. De meest voorkomende genera waren *Lactococcus* (31,2%), *Staphylococcus* (11.7%), *Serratia* (10.4%), *Acinetobacter* (9.1%), *Enterococcus* (7.8%) en *Pseudomonas* spp. (6.5%). Het overzicht van de fysisch-chemische eigenschappen van de *Pangasius* producten wordt eveneens in dit hoofdstuk besproken.

In **hoofdstuk 4** werd een studie uitgevoerd in kleine en grote bedrijven om het kwaliteitszorgsysteem te analyseren naar de performantie inzake microbiologische voedselveiligheid. Dit gebeurde met behulp van een instrument voor zelfevaluatie en een *Microbial Assessment Scheme* (MAS). Dit laatste werd in het kleine bedrijf toegepast op een productielijn waarbij gechlorineerd waswater gebruikt wordt en in het grote bedrijf op een

productielijn waar niet-gechlorineerd waswater gebruikt wordt. De resultaten demonstreren dat de microbiële veiligheid en kwaliteit van de producten van het kleine bedrijf niet gegarandeerd kunnen worden omdat de contaminatieniveaus hoog blijven tijdens de productie. *Escherichia coli*, *Staphylococcus aureus* en *Vibrio cholerae* konden gedetecteerd worden op de handen van de arbeiders, vooral in de verpakkingsunit. De aanwezigheid van *Listeria monocytogenes* (1 positief resultaat op 9 monsters) in het eindproduct is dus waarschijnlijk te wijten aan onvoldoende hygiënepraktijken. Daarentegen werden op de eindproducten van het grote bedrijf slechts lage aantallen psychotrofe bacteriën (*ca.* 3 log kve/g) en *E. coli* (enkel detecteerbaar) teruggevonden. Bovendien waren *L. monocytogenes* en *V. cholera* afwezig in alle geanalyseerde monsters. In dit hoofdstuk worden ook de resultaten van de zelfdiagnose onder de loep genomen. Deze zelfdiagnose levert inzicht in de performantie van het huidig geïmplementeerde autocontrolesysteem. Problemen op het niveau van de controleactiviteiten en kwaliteitsborging in het kleine bedrijf resulteren in pangasiusfilets met een hoog risico inzake microbiologische voedselveiligheid.

In een eerste deel van **Hoofdstuk 5** werd een preliminaire evaluatie uitgevoerd om na te gaan in welke mate *Pangasius* gedecontamineerd werd in hetzelfde kleine schaal bedrijf. Aangezien chloor enkel toegevoegd werd in het begin van het wasproces en het water continu werd hergebruikt gedurende 239 min, werden het totaal psychrotroof kiemgetal, *E. coli* en coliformen op de *Pangasius* filets niet significant verwijderd ($p > 0.05$) tijdens het wassen. Dit kan verklaard worden door de snelle accumulatie van organische materie wat resulteerde in een dalend vrij chloor residu van 34.4 ± 2.9 ppm tot 7.8 ± 3.6 ppm na 24 min. Ook namen de microbiële aantallen in het waswater toe tot 5.7 (totaal kiemgetal), 3.9 (coliformen) en 3.0 (*E. coli*) log KVE/100 mL na 24 min. In een tweede deel van hoofdstuk 5 werd de disinfectie efficiëntie van chloor en perazijnzuur (PAA) op zowel het proceswater als de *Pangasius* filets getest op **laboschaal**. Wassen met chloor en PAA resulteerde in een *E. coli* reductie op de *Pangasius* die varieerde van 0-1.0 en 0.4-1.4 log CFU/g respectievelijk, terwijl lagere reducties van totaal psychrotroof kiemgetal, melkzuurbacteriën en coliformen bereikt werden op *Pangasius* filets. Daarentegen, in vergelijking met PAA, reageerde chloor snel weg in het waswater. Bijvoorbeeld, 53-83% van het chloor en enkel 15-17 % van het PAA reageerde weg na wassen gedurende 40 s (Chemische Zuurstof Vraag (CZV) = 238.2 ± 66.3 mg O_2/L). Daarom kan PAA dienen als alternatief waterdesinfectans, vooral in bedrijven waar het waswater hergebruikt wordt om meerdere batches te wassen. Maar, de hogere kost van PAA zal in rekening gebracht moeten worden. Bij toepassing van het (goedkopere) chloor, zullen

het residu aan chloor, pH en de CZV tijdens het desinfectieproces aandachtig gecontroleerd moeten worden om een optimale desinfectie te garanderen.

Gebaseerd op de screening in Hoofdstuk 5, werd in **Hoofdstuk 6** het effect van het continu doseren van chloor in het waswater geëvalueerd op het decontamineren van de *Pangasius*, de microbiële waswater kwaliteit en de sensorische kwaliteit en chemische veiligheid. In vergelijking met de controle (waar niet-gechloreerd waswater werd gebruikt), werd een hoge microbiële reductie waargenomen in het waswater bij continue dosering van chloor in het waswater. Bijvoorbeeld, de concentratie aan psychrotrofe micro-organismen in het waswater waren 2-4 log KVE/100 ml lager dan deze in niet-gechloreerd waswater na het wassen van 12 batches filets. Na het wassen van de laatste batch filets (batch 12), waren de concentratie aan totaal chloor en organische materie in het water geaccumuleerd tot 482.9 ± 17.0 ppm en 4447.5 ± 187.4 mg O_2/L respectievelijk. Daarentegen, slechts 8.9 ± 1.3 µg/L aan trihalomethanen werden gevormd in het gechloreerde waswater en geen trihalomethanen werden gedetecteerd op de gewassen *Pangasius* filets na naspoelen. Er werd besloten dat *Pangasius* filets die gewassen werden in continu gechloreerd water, veilig waren voor menselijke consumptie, en dit voor alle batches (dus ook de laatste of 12^{de} batch). Maar, de organoleptische eigenschappen van de *Pangasius* filets in de laatste batch waren na wassen in chloor onacceptabel vanwege verkleuring (verbleking) en de aanwezigheid van een chloorgeur. Daarom, volgens de sensorische data, dient het aantal batches dat gewassen wordt in continu gechloreerd water gelimiteerd te zijn tot 4 batches. Er werd ook bepaald dat de hoeveelheid organische materie en de vrij chloor residuen in het waswater sneller en eenvoudiger bepaald konden worden door het meten van de UV absorbantie (bij 249 nm) en de redox potentiaal (ORP) respectievelijk. Het continue doseersysteem blijkt een groter potentieel te bezitten in een wasproces waar meerdere batches na elkaar gewassen worden in hetzelfde water.

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PUBLICATIONS

Publications in peer reviewed A1 journals

Tong Thi, A.N, Noseda, B., Samapundo, S., Nguyen, B.L., Broekaert, K., Rasschaert, G., Heyndrickx, M., and Devlieghere, F., 2013. Microbial ecology of Vietnamese Tra fish (*Pangasius hypophthalmus*) fillets during processing. International Journal of Food Microbiology. 167(2):144-152.

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Contribution to Conferences and Symposia

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