Shrimp quality and safety management along the supply chain in Benin

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Shrimp quality and safety management along the supply chain in Benin

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Contents

Abstract

This thesis focuses on quality and safety management of tropical shrimp (*Penaeus* spp.) using Benin (West Africa) as an example of a shrimp exporting country. The entire supply chain, from fishing areas (brackish waters) to shrimp processing plants, was investigated. The steps of the chain prior to shrimp processing at the freezer plants were critical for shrimp quality and safety because of prevailing temperature abuse and inappropriate hygienic conditions. Combining culture-dependent (plate counts) and culture independent (DGGE, clone libraries analysis) approaches, it was found that bacterial concentration in shrimps was higher than that of their surrounding water and sediment. Conversely, bacterial diversity was higher in water or sediment than in shrimps. At species level, distinct bacterial communities were associated with sediment, water or shrimp samples. Spoilage evaluation of shrimps showed that during storage at 0ºC, *Pseudomonas* spp. were dominant, whereas at 7ºC and 28ºC, H_2S-producing bacteria were the dominant group of microorganisms. An empirical model predicting shrimp shelf-life as a function of constant storage temperature was developed. Isolates producing strong off-odor were identified by 16S rRNA sequencing as mainly lactic acid bacteria (LAB) and *Enterobacteriaceae* at 28ºC or 7ºC and *Pseudomonas* spp. and LAB (*Carnobacterium maltaromaticum*) at 0ºC. The fastest growing isolates namely, *Pseudomonas psychrophila* and *C. maltaromaticum* were selected for their spoilage activity and for modeling studies. *P. psychrophila* had a higher growth rate and a higher spoilage activity at 0 to 15ºC, while at 28ºC, *C. maltaromaticum* had a higher growth rate. Models predicting the growth of pseudomonads in shrimps as a function of temperature were constructed. These models were validated under dynamic storage temperatures simulating actual temperature fluctuation in the supply chain. Using different risk classification approaches, the main foodborne pathogen risks identified were *Vibrio parahaemolyticus* and *Salmonella*. The management of the risks posed by the main pathogens was addressed using different scenarios to meet the set food safety objectives. Based on quantitative and ecological studies, this thesis developed tools that can be used in decision-making regarding tropical shrimp quality and safety management.

1

Introduction and outline of the thesis

Abstract

*Food spoilage and food safety are important food quality attributes. In general, food spoilage refers to chemical or microbiological changes that occur in foods making them less enjoyable or even inedible based on sensory assessment. Safe foods are those which do not contain hazards capable of causing illness to consumers. These hazards may be of chemical origin (e.g. toxic compounds) or biological (e.g. pathogens). Microorganisms can thus affect the quality of foods by spoiling them or making them unsafe. The effect of microorganisms on food quality depends on the food properties and the conditions prevailing in the entire supply chain. The microbial community present in fishery products may depend on the ecology of these fishery products in aquatic environments. For food quality and safety management, a quantitative description of microbial kinetics can be used to develop mathematical models that predict the growth or survival of microorganisms in foods. This modeling approach should be based on the assessment of the ecology of microorganisms in foods and along the supply chain. This thesis combines quantitative and ecological studies to develop tools for tropical shrimp (*Penaeus *spp.) quality and safety management. This introductive chapter describes relevant background aspects related to the work.*

Shrimps: species, habitat, and life cycle

Shrimps belong to the decapod crustaceans, a highly diverse group of organisms with about 233 families and 2,725 genera (De Grave et al., 2009). The genus *Penaeus* (Family *Penaeidae*) is economically the most important group of shrimps (Dall et al., 1990) containing 27 species (Holthuis, 1980) (Table 1.1), including *Penaeus notialis* (Fig. 1.1A) which is one of the most commercialized species in West Africa. The black tiger shrimp (*Penaeus monodon*) (Fig. 1.1B), which was in the past absent from brackish waters (lakes and lagoons) where shrimps are mainly caught in Benin, has been found recently in Beninese brackish waters, and has become also a species of commercial importance. The origin of the invasion of Beninese brackish waters by this species has not yet been elucidated. However, it has been reported that *P. monodon*, which is native to the Indo-west Pacific is currently established in many areas including West Africa due to escapes from aquaculture (Fuller et al., 2014).

A B

Fig. 1.1 Shrimp species (A) *P. notialis* and (B) *P. monodon* of commercial importance in Benin.

Note that there is confusion in the use of the terms “shrimp” and “prawn”. Both terms are often used interchangeably. In general, penaeid shrimps (shrimps belonging to the Family *Penaeidae*) are called shrimps in America and prawns in other English-speaking countries (Holthuis, 1980). Moreover, among shrimp taxonomists, there is no unanimity on the systematic position of *Penaeus* species. Pérez-Farfante and Kensley (1997) revised the sub-genus names of *Penaeus* to the rank of genus. However, Flegel (2007) argued that it was not necessary to revise the taxonomy. In this thesis, the term “shrimp” is used and the species nomenclature used is according to Holthuis (1980).

Penaeid shrimps occupy mainly four marine basins, namely the Indo-west Pacific, the eastern Pacific, and the western and eastern Atlantic (Dall et al., 1990; Holthuis, 1980) (Table 1.1). They may display different types of life cycle. However, in general juveniles inhabit brackish waters of estuaries and coastal wetlands, while adults prefer off-shore at higher salinities and greater depths. Larvae are found in plankton-rich surface waters off-shore, with an on-shore migration as they develop (Bailey-Brock and Moss, 1992). It has been reported that part of the life cycle of penaeid shrimps caught from West African coastal brackish waters (lagoons and lakes) occurs also in the Atlantic Ocean. Adults spawn in marine waters. After oviposition, the post-larvae migrate to the brackish waters for growth to post-juvenile stage. Then, the animals return to the sea for reproductive purpose (John and Lawson, 1990; Sankare et al., 2014).

Water salinity and temperature are two important abiotic factors that influence the growth and survival of shrimps. The optimum temperature and salinity for growth of juvenile shrimp (*Penaeus merguiensis*) are 31°C and 30‰ (Staples and Heales, 1991). Ponce-Palafox et al. (1997) showed that *Penaeus vannamei* juveniles grew best at salinity of approximately 20‰ at temperatures varying between 25 and 35°C. The optimum temperature and salinity for growth of juvenile brown tiger shrimps *Penaeus esculentus* were found to be 30°C and 30‰ (O’Brien, 1994).

Table 1.1

Penaeus species and their native habitats (Holthuis, 1980). The most important species in Benin *P. notialis* and *P. monodon* are indicated in bold

Genus	Sub-genus	Species	Native habitat
Penaeus	*Farfantepenaeus*	*aztecus*	Western Atlantic (Atlantic coast of U.S.A. from Massachusetts to Texas; east coast of Mexico from Tamaulipas to Campeche)
		brasiliensis	Western Atlantic coast of America from North Carolina (U.S.A) to Rio Groande do Sul (Brazil); Bermuda; West Indies
		brevirostris	Eastern Pacific (Sinaloa (Mexico) to northern Peru and the Galápagos Islands)
		californiensis	Eastern Pacific (California to Paita (Peru))
		duorarum	Western Atlantic (Bermuda; Atlantic coast of the U.S.A. from Maryland to Texas; east coast of Mexico (from Tamaulipas to Quintana Roo)
		notialis	Eastern Atlantic (West African coast from Mauritania to Angola); western Atlantic (Greater Antilles from Cuba to the Virgin Islands); Atlantic coast of middle and South America
		paulensis	Western Atlantic (southern Brazil to Argentina (38°30'S))
		subtilis	Western Atlantic (West Indies from the Greater Antilles south; Atlantic coast of Central and South America from Honduras to Brazil (Rio de Janeiro)
	Fenneropenaeus	*chinensis*	Indo-west Pacific (Korea; China; Hong Kong)
		indicus	Indo-west Pacific (East and South East Africa to South. China, New Guinea and New Australia)
		merguiensis	Indo-west Pacific (from the Persian Gulf to Thailand; Hong Kong; the Philippines Indonesia; New Guinea; New Caledonia; New Australia)
		penicillatus	Indo-west Pacific (from Pakistan to Taiwan and Indonesia)
	Litopenaeus	*occidentalis*	Eastern Pacific (Off Chiapas (Mexico) to Peru)
		schmitti	Western Atlantic (Greater Antilles from Cuba to Virgin islands), Atlantic coast of Central and South America from British Honduras to South Brazil
		setiferus	Western Atlantic (east coast of U.S.A. from New Jersey to Texas); east coast of Mexico from Tamaulipas to Campeche; Gulf of Mexico
		stylirostris	Eastern Pacific (from Baja California (Mexico) to Peru)
		vannamei	Eastern Pacific (from Sonora, Mexico, South to northern Peru)
	Marsupenaeus	*japonicus*	Indo-west Pacific (from the Red Sea, East and South East of Africa to Korea, Japan and the Malay Archipelago; also reported from Fiji, eastern Atlantic, eastern Mediterranean (south coast of Turkey)
	Melicertus	*canaliculatus*	Indo-west Pacific, extensive area (from South East Africa to Taiwan, the Malay Archipelago and Polynesia)
		kerathurus	Eastern Atlantic (from the south coast of England to Angola, and the entire Mediterranean)
		latisulcatus	Indo-west Pacific (Red Sea and South East Africa to Korea, Japan, the Malay Archipelago and Australia)
		longistylus	Indo-west Pacific (South China sea, Malaysia, North West, North and North East Australia, Lord Howe island)
		marginatus	Indo-west Pacific (East Africa and Madagascar to Singapore, Indonesia, Japan, Cocos islands, and Hawaiian Islands)
		plebejus	Indo-west Pacific: East Australia (from South Queensland to Victoria)
	Penaeus	*esculentus*	Indo-west Pacific western, North and East Australia (from Shark Bay to Central New South Wales)
		monodon	Indo-west Pacific (East and South East Africa and Pakistan to Japan, the Malay Archipelago and northern Australia)
		semisulcatus	Indo-west Pacific (Red Sea, East and South East Africa to Japan, Korea, the Malay Archipelago and northern Australia); eastern Atlantic; eastern Mediterranean (Egypt, Israel, Lebanon, Syria and southern Turkey)

While a small variation of temperature can greatly affect the growth of shrimps (Dall et al., 1990; O'Brien, 1994; Ponce-Palafox et al., 1997), most of penaeid shrimps are euryhaline i.e. they are able to adapt to a wide range of salinities (Dall et al., 1990; Kaeodee et al., 2011). No reports were found on the optimum temperature and salinity for the growth of *P. notialis*. However, the temperature of brackish waters from where this species is caught in Benin ranges between 25 and 33°C, and the salinity, from nearly 0 to 30‰ (Laleye et al., 2003; Maslin and Pattee, 1989). The salinity is nearly 0‰ when the rivers that bring freshwater to the brackish waters flood (usually September-December). During this period, shrimps are absent from the brackish waters, forcing shrimp processing plants to close their premises temporarily.

World shrimp production and global trade

World production of shrimps has increased with the increasing demand for shrimps in the world market. Global shrimp production of approximately 4.3 million metric tons (mmt) in 2002 showed an increase of 79% to reach approximately 7.7 mmt in 2012 (Fig. 1.2).

This increase in global shrimp production was mainly due to shrimp aquaculture (farming), which has emerged consistently. As shown in Fig. 1.2, the proportion farmed shrimps increased from 34% to 56% of shrimps produced globally from 2002 to 2012. Countries that contribute most to global shrimp aquaculture are in Asia (Thailand, India, Vietnam, and China) and Latin America (Ecuador, Nicaragua Honduras, Peru) (Globefish, 2014).

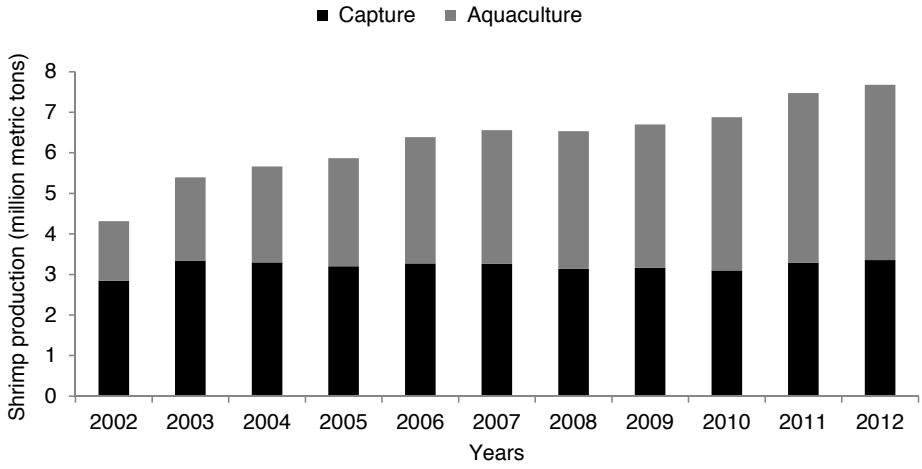

Fig. 1.2 Global shrimp production (FAO, 2012).

Shrimp aquaculture is not yet practiced in Benin. About 3,000 tons of shrimps are captured annually from brackish waters, namely Lake Nokoue, Lake Aheme and Lagoon of Porto-Novo (Le Ry et al., 2007). Shrimps (wild and farmed) are the most important seafood commodity traded in global markets (SFP, 2014). In general, shrimps are exported from developing countries to the developed world. The European Union (EU) is the largest shrimp importing market with approximately 800,000 tons per year since 2008 (Fig. 1.3). Within the EU, Spain, France and Denmark are the most important shrimp importing countries (Fig. 1.4).

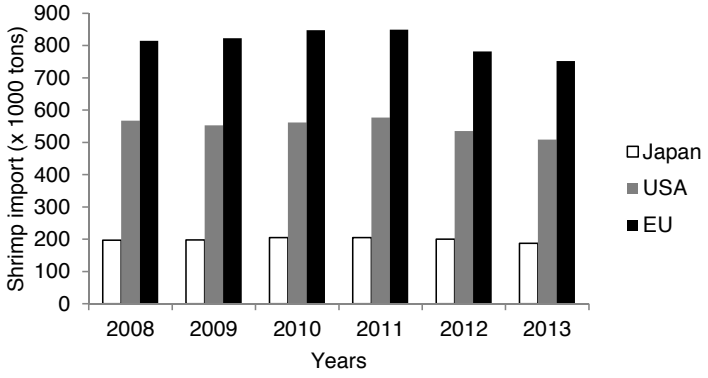

Fig. 1.3 Shrimp import (in volume) in Japan, United States of America (USA) and the European Union (EU) (Globefish, 2014).

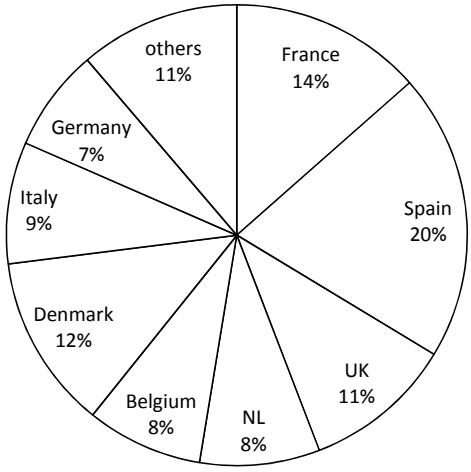

Fig. 1.4 Main shrimp importers in the European Union.
The percentage represents the proportion of the volume of shrimps imported by each country over the period 2008-2013 (source: Globefish, 2014).
Legend: NL = the Netherlands, UK = the United Kingdom.

Benin exports its shrimps mainly to Belgium, Spain and France as raw frozen shrimps (Dabadé et al., 2014). The volume exported reached 700 tons in 2002 (Le Ry, 2007). However, after a ban on export to the EU that occurred from 2003 to 2005 the exported volume has decreased.

Import regulations and EU legislation about seafood

Like the shrimp sector, global seafood trade is increasing (Baylis et al., 2011). Consumers and authorities have become more aware of food safety risks and expect safe handling of food (Beulens et al., 2005; Ko, 2010; Unnevehr, 2000). Therefore, food safety standards have become an increasingly prominent issue for global trade in agricultural products (Jaffee and Henson, 2004). The European Commission's Directorate-General for Health and Consumer Protection (DG SANCO) is responsible for food safety in the EU. Its import rules are to ensure that the level of safety from imported seafood products is at least of the same level as that of seafood products

produced within the EU. Therefore, the EU has implemented requirements that seafood exporting countries must fulfill, in order to have access to Europeans markets. European legislation for seafood products can be divided into general food safety requirements, which are applicable to all food products marketed in the EU, and requirements established specifically for fish products. The **regulation EC/178/2002** known as the general food law, establishes the general principles and requirements of food law, lays down procedures on matters of food safety, and establishes common principles and responsibilities for food and feed business operators at all stages of production, processing and distribution. It also covers the concept of traceability defined as a compulsory rule to trace and follow food, feed and ingredients through all stages of production, processing and distribution. **Regulation EC 852/2004** of the European Parliament and of the Council of 29 April 2004 deals with the hygiene of foodstuffs. It includes general and technical requirements for primary production, including Hazard Analysis Critical Control Points (HACCP). **Regulation EC 853/2004** and its amendments lay down specific hygiene rules for food of animal origin. The regulation includes fish-specific requirements on equipment and facilities of fishing, factory and freezer vessels, landing sites, processing establishments and on operational processes, storage and transport of fishery products, and production areas of live bivalve mollusks. **Regulation EC 854/2004** lays down specific rules for the organization of official controls on products of animal origin intended for human consumption. **Regulation EC 882/2004** is related to the official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.

To be eligible to export seafood products to the EU market, exporting countries must have legislation and controls for the fishery sector that are equivalent to the EU legislation. Official guarantees that the seafood products to be exported comply with food safety standards must be given by the Competent Authority of the exporting country i.e. the central authority competent for the organization of official control (see Regulation EC 882/2004). The Competent Authority in Benin is the Directorate of Fishery. Establishments willing to export seafood products to the EU market must obtain an agreement from the Competent Authority of their country. To ensure that exports of seafood products into the European Union can take place, the Food and Veterinary Office (FVO) of the European Commission carries out missions to exporting countries to assess the hygienic conditions under which seafood products, intended for export to European Union, are produced.

Ban on exported seafood to EU

The importation of seafood from a country to the EU can be banned when the production and export conditions of seafood products in that country do not comply with EU food safety and quality standards. The ban may be imposed by the European Commission or by the exporting country itself (self-ban) as a precautionary action. For example, in 1997, the European Commission banned imports of fishery products from Bangladesh into the EU (EEC, 1998). From 1997-2000, the European Commission imposed repeated bans on seafood products imported from Uganda, Kenya, and Tanzania to Europe (Henson and Mitullah, 2004; Ponte, 2007). After the visit of FVO experts in Malaysia who found shortcomings related to seafood quality and safety management, the Malaysian government decided in 2008 to self-ban the export of seafood products to the EU market (Alavi, 2009; Golnaz et al., 2012). In Benin, the FVO experts found in 2002 that the shrimp collection network from the landing sites to shrimp processing plants was not well organized and that the HACCP plans at the shrimp processing plants were not well documented. In addition, Benin lacked the legal basis to ensure shrimp quality and safety (FVO, 2003). In reaction to the results of the FVO technical inspection, the national government decided to self-ban shrimp export as a precautionary action. The ban was lifted in 2005. However, during a second visit in 2009, the FVO experts observed that only the Beninese legislation could be considered equivalent to the European Union feed and food law, whereas most of the deficiencies related to shrimp quality and management remained.

The ban on exports of fishery products to the European Union had a significant impact at both the macro and micro economic levels. Indeed, the integration of developing countries in global trade is generally believed to stimulate economic growth, thus to reduce poverty in those countries (Dollar and Kraay, 2002; Irwin

and Tervio, 2002). For example, the European Commission ban on seafood imports into the European Union from Bangladesh in 1997 was estimated to cost the Bangladesh shrimp processing sector about 15 million US dollars in lost revenues from August to December 1997 (Cato and Dos Santos, 1998). In Benin, the ban resulted in 60,000 lost jobs and about 10 million Euros in lost revenues from 2003 to 2006 (Le Ry, 2007).

Approaches to detect microorganisms

Unlike other crustaceans (crabs, lobsters), which can be kept alive until processing, shrimps die soon after being caught, and they are often contaminated with bacteria from their endogenous microflora as well as from the mud trawled up with them (Adams and Moss, 2000). It is therefore of importance to detect the types of microorganisms associated with shrimps and their habitats for shrimp quality and safety management. Two approaches for microbiological detection can be used: the culture-dependent approach and the culture-independent approach.

Culture-dependent approach

One of the most sensitive methods of detecting the presence of a viable bacterium is to culture it on the surface or within the matrix of a medium solidified with agar (Adams and Moss, 2000). This technique, known as the plating method, may require a pre-enrichment step to increase the concentration of injured but viable bacteria to a detectable level (Zhao and Doyle, 2001). Microbiological media can be selective for a limited group of species, and even a single species, or be of general purpose, allowing the growth of the widest possible range of organisms (Adams and Moss, 2000). Plate count agar (PCA) is a non-selective medium widely used to enumerate total counts in food. In seafood products however, it has been reported that PCA underestimates bacterial counts in comparison with other media namely iron agar (IA), marine agar (MA), and Long and Hammer (LH) agar (Broekaert et al., 2011; Gram, 1992; Yesudhason et al., 2014). Conventional methods for the identification of microorganisms rely on a variety of morphological, physiological and biochemical tests (De Boer and Beumer, 1999). Molecular methods are now used as an alternative or complement to conventional methods. Full and partial 16S rRNA sequencing methods have proved useful to identify bacterial isolates (Petti et al., 2005). The 16S rRNA gene is universal in bacteria, and many sequences of this gene have been deposited in public databases, such as the GenBank, which can be used to compare unknown sequences (Clarridge, 2004). Although widely used to enumerate microorganisms, the traditional culture-dependent analytical approach has its limitations when unravelling the natural microbiota ecosystem because not all microorganisms present can grow on the selected media and under the incubation conditions used.

Culture-independent approach

Culture-independent approaches rely on the extraction of DNA or RNA directly from samples, followed by amplification by the polymerase chain reaction (PCR) of the genes of interest and their identification either by sequencing or direct visualization in appropriate gel systems (Xu, 2006). Introduced into microbial ecology by Muyzer et al. (1993), denaturing gradient gel electrophoresis (DGGE), has been widely used to investigate microbial diversity. It is based on the separation of PCR amplicons of the same size but different sequences in polyacrylamide gels containing a linearly increasing gradient of denaturants (urea and formamide) (Muyzer et al., 1993). Initially used in environmental microbiology, PCR-DGGE was later introduced into food microbiology and its applications in this field have been reviewed (Ercolini, 2004).

Another culture-independent approach widely used in microbial community studies is the so-called 16S rRNA clone library analysis. The approach is based on PCR-amplification of 16S rRNA genes from genomic DNA directly extracted from samples, ligation of purified PCR products into an appropriate vector system, transformation of the recombinant vectors in a suitable bacterial host, and Sanger sequencing of the recombinant clones (Carlos et al., 2012; Handelsman, 2004). The Sanger sequencing, also known as terminator sequencing, is

based on primer extension with chain-terminating dideoxynucleotides (Sanger et al., 1977). The method has undergone major improvements during the years to make it a robust technique that has been used for the sequencing of various bacterial, archeal, and eukaryotic genomes (Ronaghi, 2001). With the 16S rRNA clone library approach, each individual colony represents one 16S rRNA gene, limiting most studies to few (100-500) sequences per sample (Carlos et al., 2012). Therefore, in surveys of highly diverse ecosystems, it is believed that the approach fails to detect the rare members of the community (Youssef et al., 2009). Fortunately, next-generation sequencing (NGS) technologies recently introduced into microbial communities analysis, offer the possibility to deliver a large amount of sequence data cheaply (Metzker, 2009). With NGS technologies, sequences are not generated through vector-based cloning procedures but from fragmented libraries of a specific genome (i.e. genome sequencing); from a pool of cDNA (complementary DNA) library fragments generated through reverse transcription of RNA molecules (i.e. RNAseq or transcriptome sequencing); or from a pool of PCR amplified molecules (i.e. amplicon sequencing) (Shokralla et al., 2012). NGS technologies, namely 454 pyrosequencing or Illumina, have been used in microbial diversity analysis to generate sequences directly from a pool of amplified 16S rRNA genes and results showed better bacterial diversity coverage than the conventional 16S rRNA clone library (Bartram et al., 2011; Youssef et al., 2009). However, in a comparative study of the Norway lobster (*Nephrops norvegicus*) gut bacterial communities using 16S rRNA cloning and 454 pyrosequencing Meziti and Kormas (2013) found that both types of analysis provided similar results in terms of bacterial diversity coverage. As previously reported, the choice of the technique or approach to be used to unravel microbial diversity depends on the research question being answered and the level of detail required (Hamady and Knight, 2009).

Microbiota of shrimps

Like every food product, the composition of the microbiota of shrimps is determined by their initial contamination, their processing parameters and subsequent storage conditions (Vanderzant et al., 1971). Plate count techniques revealed that in general, tropical shrimps carry higher initial numbers of bacteria, 10^5-10^6 CFU/g, than cold-water species, 10^2-10^4 CFU/g (ICMSF, 2005).

Microorganisms are the major cause of spoilage of seafood products (Gram and Dalgaard, 2002). However, only a few members of the initial microbial community of seafood products, the so-called specific spoilage organisms (SSOs) really contribute to the seafood spoilage (Dalgaard, 1995). Shrimp spoilage organisms vary according to shrimp species, their geographic origins, the types of processing they undergo, and their storage conditions. In fresh shrimps (*Penaeus aztecus*) harvested from coastal waters of Georgia, the main spoilage organisms during storage at 0°C were found to be *Acinetobacter* spp. (Heinsz et al., 1988). The dominant spoilage organisms of fresh Pacific coast shrimp (*Pandalus jordan*i) were *Pseudomonas* spp. and *Moraxella* spp. at low storage temperatures (0-11°C), and *Moraxella* spp. and *Proteus* spp. at high storage temperatures (17-22°C) (Matches, 1982). *Enterobacteriaceae* were found to be the main spoilage organisms of pink shrimp (*Parapenaeus longirostris*) caught from the Algarve coast (Portugal) during storage at room temperature (22°C) (Mendes et al., 2002). Chinivasagam et al. (1996) found that *Pseudomonas fragi* was the main spoiler of iced-stored tropical shrimps, and *Shewanella putrefaciens* was the dominant microorganism in tropical shrimps stored in ice slurry. *Pseudomonas* spp. were the dominant spoilage organisms of fresh Indian shrimps (*Penaeus indicus*) stored at 0°C (Jeyasekaran et al., 2006). Mejlholm et al. (2005) reported that *Brochothrix thermosphacta* and *Carnobacterium maltaromaticum* were responsible for sensory spoilage of cooked and peeled cold-water shrimps (*Pandalus borealis*) in modified atmosphere packaging (MAP) conditions. *Enterococcus* spp. and *Carnobacterium* spp. were the dominant members of spoilage associations of cooked and brined modified atmosphere packed cold or warm water shrimps stored at high (25°C) and low (0°C) temperatures, respectively (Dalgaard et al., 2003). The main spoilage organisms of cooked pink shrimps caught

from the south coast of Spain during storage at 2°C under air condition were *Pseudomonas* spp. (Martinez-Alvarez et al., 2009).

In addition to spoilage organisms, various pathogenic organisms are associated with shrimps. Like other seafood products, shrimp is high on the list of foods transmitting disease (Huss et al., 2000). *Vibrio parahaemolyticus*, the leading cause of seafood-associated gastroenteritis (Haley et al., 2014; Martinez-Urtaza et al., 2013) can be present in shrimps. Xu et al. (2014) found a prevalence of 37.7% (*n*=273) in shrimps from Chinese retail markets. The prevalence of *Vibrio parahaemolyticus* in shrimps collected from shrimp retail outlets in the south-western part of Iran ranged between 4% (*n*=75, winter samples) and 19% (*n*=75, summer samples) (Zarei et al., 2012). Dileep et al. (2003) found that 80% (*n*=10) of shrimps from India were positive for *Vibrio parahaemolyticus*. The prevalence of *Vibrio parahaemoltyticus* was 22% (*n*=120) in shrimps from Nigeria (Eja et al., 2008) and 76% (*n*=62) in shrimps from Thailand (Wong et al., 1999). Despite this high prevalence of *Vibrio parahaemolyticus*, only strains producing thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) are virulent (Nishibuchi and Kaper, 1995). In addition to *Vibrio parahaemolyticus*, the microbial flora of shrimp may also be composed of other human pathogenic *Vibrio* namely *Vibrio cholerae* and *Vibrio vulnificus*. Wong et al. (1995) reported a prevalence of 13% (*n*=40) of *Vibrio cholerae* in frozen shrimps from Taiwan. The prevalence of *Vibrio vulnificus* in warm-water shrimps imported into Denmark was reported to be 7% (*n*=47) (Dalsgaard and Hoi, 1997).

Several authors have reported also the prevalence of *Salmonella* in shrimp. Koonse et al. (2005) found that 1.6% of 247 shrimp samples from aquatic farms were positive for *Salmonella*. In India, Kumar et al. (2009) detected *Salmonella* in 27% (23/86) of shrimp samples collected from fishing harbors and fish markets. However, authors are not unanimous that like *Vibrio* species, *Salmonella* is part of the natural flora of the shrimp culture environment. The findings of Dalsgaard et al. (1995) were not in agreement with those of Reilly (1987) and Reilly and Twiddy (1992) who suggested that *Salmonella* is part of the natural flora of tropical brackish water environment. As a result, Dalsgaard et al. (1995) believed that contamination of shrimps with *Salmonella* may be due to poor standards of hygiene during processing. While the prevalence of *Salmonella* in shrimps is sometimes high, human salmonellosis associated with the consumption of seafood occurs rarely (Fell et al., 2000). In fact, strains isolated from most human salmonellosis appear to be different from those found in harvested products (Reilly and Twiddy, 1992).

Aeromonas spp. known to be ubiquitous in aquatic environments (Monfort and Baleux, 1991) have been detected in 18% of shrimp samples (*n*=278) in India (Vivekanandhan et al., 2005). *Aeromonas* spp. were also found in 17% (*n*=12) of shrimp from Finland (Hanninen et al., 1997).

In addition to indigenous bacteria, shrimps like other seafood products may be contaminated by enteric bacteria due to fecal contamination (*Escherichia coli*, *Shigella* spp. etc.) or during processing (*Listeria monocytogenes*, *Staphylococcus aureus* etc.) (Feldhusen, 2000).

From bacterial community point of view, *Proteobacteria*, specifically members of the class *Gammaproteobacteria* (*Vibrionaceae*, *Pseudomonaceae*, *Enterobacteriaceae*) were found to be the most abundant microorganisms in the intestinal tract of Chinese shrimp (*Fenneropenaeus chinensis*) (Liu et al., 2011), Pacific white shrimp, *Penaeus vannamei* (Johnson et al., 2008), black tiger shrimp (*P. monodon*) (Chaiyapechara et al., 2012; Rungrassamee et al., 2014), and banana shrimp, (*Penaeus merguiensis*) (Oxley et al., 2002). *Firmicutes* members (namely *Bacillales*, *Clostridiales*, lactic acid bacteria) have also been reported to be associated with the intestinal tract of some shrimp species namely, *F. chinensis* (Liu et al., 2011), *P. monodon* (Chaiyapechara et al., 2012; Rungrassamee et al., 2014). However, the occurrence of *Firmicutes* associated with shrimp is generally low. They were not even detected in the intestinal tract of *P. merguiensis* (Oxley et al., 2002) and *P. vannamei* (Johnson et al., 2008).

Quantitative microbiology

Factors affecting the growth, survival or inactivation of microorganisms in foods are mainly the properties of the food (intrinsic factors), the environmental conditions (extrinsic factors) and the processing factors. Quantitative knowledge can be gathered to understand the response of microorganisms to intrinsic factors (e.g., water activity and pH) and extrinsic factors (e.g. temperature and atmosphere) and to build a database of knowledge (Baranyi and Roberts, 1995). Quantitative description of microbial kinetics can be used to develop mathematical models that predict the growth or survival responses of microorganisms in response to environmental factors. This modeling approach is also termed "quantitative microbiology" or "predictive microbiology". It is based on the assumption that the responses of microorganisms to environmental factors are reproducible, and as a result, past observations can be used to predict future responses of microorganisms to environmental factors (Ross and McMeekin, 1994). Quantitative microbiology can be used as a tool to improve food quality and safety. Indeed, predictive models offer an alternative to traditional microbiological assessment of food quality and safety such as shelf-life tests and challenge tests (McMeekin et al., 1992). They can be applied throughout the whole supply chain, to determine what may happen in many different scenarios (Perez-Rodriguez and Valero, 2013; Zwietering et al., 1996) and therefore, they can be used to support decisions in food quality and safety management (Zwietering et al., 1996). However, predictive models may sometimes generate data that are very different from the observed ones. Thus, it is of great importance to validate the developed models to determine the level of confidence one can have while using them (Ross, 1996).

Microbiological risk assessment (MRA)

According to Codex Alimentarius, risk assessment is a scientifically based process consisting of the following steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (CAC, 1999). It enables the identification of hazards associated with foods and the estimation of the risk they may pose. It is becoming increasingly important for the control of food safety (Zwietering and Van Gerwen, 2000). A hazard is defined as a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect (CAC, 1999). In microbial risk assessment, hazards refer to pathogenic microorganisms or to their toxins. **Hazard identification** is the first essential step in the risk assessment as well as in Hazards Analysis and Critical Control Points (HACCP). Utmost care must be taken during this step to avoid overlooking hazards that in the end will potentially cause diseases. A good combination of literature knowledge and experts' knowledge is required for hazard identification (Van Gerwen et al., 1997). **Hazard characterization** consists of qualitatively and/or quantitatively evaluating the nature of the adverse health effects associated with biological, chemical and physical agents that may be present in food. For the purpose of microbiological risk assessment the concerns relate to microorganisms and/or their toxins (CAC, 2004). In MRA, this step describes the effects of the hazard (microorganism or toxin) and establishes the dose-response relationship. When establishing a dose-response relationship it is important to specify the different end points, such as infection or illness (CAC, 1999; Zwietering, 2005). **Exposure assessment** is defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical and physical agents via food as well as exposures from other sources if relevant (CAC, 2004). In MRA, exposure assessment requires knowledge on the level of contamination with the microorganism or toxin at the time of consumption as well as the food intake, including consumption patterns (Sumner et al., 2004; Zwietering and Van Gerwen, 2000). **Risk characterization** is defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment (CAC, 2004). This step integrates all the information of the previous steps to generate an output known as the risk estimate.

MRA can be qualitative, semi-quantitative or quantitative. Qualitative risk assessments involve the descriptive treatment of information to estimate the magnitude of risk for example as high, low or medium and

the impact of factors affecting risk (Fazil, 2005; Sumner et al., 2004). Quantitative assessments work with numerical data to estimate the number of people that is expected to get infected or ill from a particular product (Fazil, 2005; Sumner et al., 2004). Semi-quantitative risk assessment is known as qualitative risk assessment in which some quantitative data are used. In semi-quantitative risk assessment, the risk is often estimated in a specific range, 0-100, for example. However, it should be noted that in reality, there are no sharp lines defining these categories of risk assessment (Bassett et al., 2012). The choice of one category depends on the type of data available, time, personnel, and resources (Coleman and Marks, 1999). It is admitted that a quantitative approach of risk assessment increases the flexibility, acceptability, objectivity and power of the decisions made (Fazil, 2005).

Predictive microbiology can be used as a tool for Quantitative Microbiological Risk Assessment (QMRA). For example, the hazard characterization step of QMRA relies mainly on dose-response models. A microbiological dose-response model that describes the probability of a specified response from exposure to a specific pathogen (or its toxins) in a specified population as a function of the ingested dose can be established (Bassett et al., 2012). Predictive models can also be used in the exposure assessment step to estimate changes in microbial concentration (or time to toxin production) as a function of the food and environmental conditions at different stages along the food chain (Bassett et al., 2012; Klapwijk et al., 2000). According to Brul et al. (2007), predictive microbiology will continue to support the full potential of QMRA.

Software tools used in MRA have been reviewed (Bassett et al., 2012). Among them, Risk Ranger has been widely used to rank and prioritize risks. From the answers provided to 11 questions, Risk Ranger returns a risk rating on a logarithmic scale of 0 to 100. Zero represents no risk and 100 represents every member of the population eating a meal which contains a lethal dose of the hazard every day (Sumner et al., 2005).

Outline of the thesis

In the recent past, the shrimp sector played a vital role in the Beninese economy. However, the ban on exports to the European Union due to lack of compliance with food safety standards prevented the sector from thriving. This thesis is part of a project involving three interdisciplinary PhD research projects aiming at determining conditions that will enable a stable access of Beninese shrimps to the international market. To achieve this aim, international trade law and marketing issues are addressed by other PhD research projects, and this thesis deals with shrimps microbiological quality and safety. The aim was to develop an approach to determine main quality and safety aspects of shrimp throughout the supply chain in Benin and to determine efficient interventions in the supply chain that can improve shrimp quality and safety.

Therefore, the entrire supply chain, from fishing areas to shrimp processing plants, was investigated to determine technical and also social aspects of relevance for better management of food safety and quality along the chain. This investigation, described in **chapter 2**, also enabled us to collect data on time and temperature regimes along the chain, that were used to evaluate relevant scenarios to determine growth of pathogenic and spoilage organisms.

In **chapter 3**, we used culture-dependent and culture-independent methods to determine bacterial communities and concentrations of groups of microorganisms in freshly caught shrimps and their surrounding water and sediment. This study allowed us to establish the relation between bacterial diversity of sediment, water and shrimps. It also provided us with the variation in initial bacterial concentration of shrimps that was useful for scenarios to predict growth of microorganisms.

For seafood quality management, it is of importance to identify the main organisms that produce sensory defects. **Chapter 4** addresses shrimp spoilage characteristics with emphasis on the identification of potential spoilage organisms. In this chapter, an empirical model that predicts the shelf-life of shrimp as a function of constant storage temperatures was developed. However, this model did not yet take into account the variation in initial microbial concentration, which can influence shrimp shelf-life. Furthermore this model can

only be used for prediction under constant storage temperatures, which actually will not occur in the supply chain. These aspects were addressed in chapter 5.

Chapter 5 deals with a comparative study of the spoilage activity of two main spoilage organisms identified in chapter 4. Models predicting the shelf-life of cooked and fresh shrimps under dynamic temperature regimes were developed. To validate the models, two scenarios simulating temperature fluctuation in the field, recorded during the investigation presented in chapter 2, were used. The models developed in chapter 5 integrate initial microbial concentration and they can be used as an important tool to support decisions in shrimp quality management.

Regarding shrimp safety management, **chapter 6** combines different approaches to determine the main foodborne pathogens risks. Thereafter, quantitative microbiology was used to predict the growth of the most important pathogens under dynamic storage temperatures simulating temperature fluctuation in the shrimp supply chain as well as their inactivation under various temperatures. The management of the risks posed by the most important pathogens was addressed using different scenarios to meet the set food safety objectives.

Finally, in **chapter 7**, the findings of this thesis are discussed and placed in perspective with suggestions for future research. Relevant topics to shrimp quality and safety that were not described in the previous chapters were also discussed.

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2

Quality perceptions of stakeholders in Beninese export-oriented shrimp chain

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Abstract

In recent years, Beninese shrimp sector has faced a ban on export to the European Union due to lack of compliance with food safety standards. The present study aimed at obtaining insight in the factors that determine shrimp quality and safety in Benin. A survey was conducted to investigate the relationship between stakeholders, the conditions under which shrimps are handled at fishing areas and processed at shrimp plants, and the stakeholders' perception of quality. A total of 325 fishermen, 128 intermediate traders, 12 collectors, and 3 shrimp processing plant managers were interviewed face to face. The results showed that various specific relations existed between the stakeholders. For example, loyalty was ensured by family relationship or incentives were provided to ensure a supply of shrimps between stakeholders. Shrimp handling practices during the steps prior to shrimp processing at the plants were not in agreement with the requirements of the European Regulations. For example, shrimps were kept at ambient temperature (28°C±1°C) by 94.1% of fishermen and 60.9% of intermediate traders. Shrimps were also stored in inappropriate holding containers and washed with nonpotable water. Fishermen, intermediate traders, and collectors considered shrimp size and texture their priority quality attributes, whereas plant managers considered shrimp appearance (freshness) and texture their priority quality attributes. This survey demonstrated that the steps prior to shrimp processing at the plants are the critical steps for shrimp quality and safety because of temperature abuse and inappropriate hygienic conditions. There is a need to communicate and provide incentives for the stakeholders in the first part of the chain to give priority to shrimp freshness. Moreover, training in Good Fishing Practice, safe food handling practices and evaluation of compliance with the practices through monitoring will contribute to a better shrimp quality and safety management.

Introduction

In recent years, food safety standards have become an increasingly prominent issue for global trade in agricultural and food products (Jaffee and Henson, 2004). Consumers and authorities have become more aware of food safety risks and expect safe handling of food (Beulens et al., 2005; Ko, 2010; Unnevehr, 2000). To ensure that imports of fishery products into the European Union can take place, the Food and Veterinary Office (FVO) of the European Commission has sent missions to all exporting countries to assess the hygienic conditions under which fishery products intended for export to the European Union were produced. In 2002, the FVO experts found that, in Benin, the shrimp collection network from the landing sites to the processing plants was not well organized and that the HACCP plans at the plants were not well documented. In addition, Benin lacked the legal basis to ensure shrimp quality and safety (FVO, 2003). In reaction to the results of the FVO technical inspection, the national government decided to self-ban shrimp export as a precautionary action. Shrimps were one of the most exported products from Benin generating jobs and revenues; the ban resulted in 60,000 lost jobs and about 10 million Euros in lost revenues from 2003 to 2006 (Le Ry et al., 2007). The ban was lifted in 2005. However, during their second visit in 2009, the FVO experts observed that only the Beninese legislation could be considered equivalent to the European Union feed and food law; the deficiencies related to shrimp quality management remained.

An important strategy for developing countries to reduce poverty is to take advantage of international trade (Lankes, 2002). Therefore, Benin should develop and promote quality improvement, spanning the entire supply chain to meet customers' expectations. The food chain may consist of several independent economic stakeholders, but the success of the final product depends on the concerted efforts of all stakeholders throughout the chain (Ottesen, 2006). Therefore, the aim of the present work was to obtain insight in the factors determining shrimp quality and safety in Benin by exploring the entire supply chain. Such an investigation is necessary to reveal the shortcomings related to shrimp quality and safety in Benin. The objectives of the present work were (i) to investigate the structure of the Beninese export-oriented shrimp chain and the relationship between stakeholders; (ii) to determine the conditions under which shrimps are handled at fishing areas and processed at shrimp plants, and (iii) to determine stakeholders' perception of quality.

Materials and Methods

Sampling of informants

The survey was conducted in the municipalities located around Lakes Nokoue and Aheme, and the lagoon of Porto-Novo (Fig. 2.1), which are the main shrimp fishing areas in Benin. Shrimp freezing plant managers and three other groups of stakeholders, namely fishermen, intermediate traders, and collectors, who are involved in shrimp transport to plants, were surveyed. To compute the sample size n of each group of stakeholders to be interviewed, the following formula (Eq. (2.1)) was used (Dagnelie, 1998):

$$n = \frac{4p(1-p)}{d^2} \tag{2.1}$$

where n is the total number of stakeholders in the group of concern to be interviewed; d is the expected error margin in the conclusion, which was fixed at 0.05, and p is the proportion of the group of stakeholders of concern among the total number of fishery stakeholders (fishery community including shrimp fishers, other fishermen, fishery products traders, fishery products processors) living in the area studied, which could be derived from the results of the census on fisheries carried out in Benin in 2006. For instance, the total number of fishery stakeholders (fishery community) living in the area studied was 29,559. Among them, 8,485 were shrimp fishers, 2,586 were shrimp intermediate traders, and 150 were shrimp collectors. A total of 328 fishermen, 128 intermediate traders, 12 collectors (instead of 9 generated by the formula) and the managers of all three Beninese freezing plants were interviewed. The numbers of interviewed fishermen, intermediate traders and collectors in each municipality were proportional to its number of fishery stakeholders and were calculated as follows:

$$n_i = n \cdot k_i \quad (2.2)$$

where n_i is the number of stakeholders in the group of concern to be interviewed in this municipality, n is the total number of stakeholders in the group of concern to be interviewed for the study (for example n=328 in case of fishermen), and k_i, the proportion of the number of stakeholders in the group of concern of a municipality i among the total number of stakeholders in the group of concern living in the area studied.

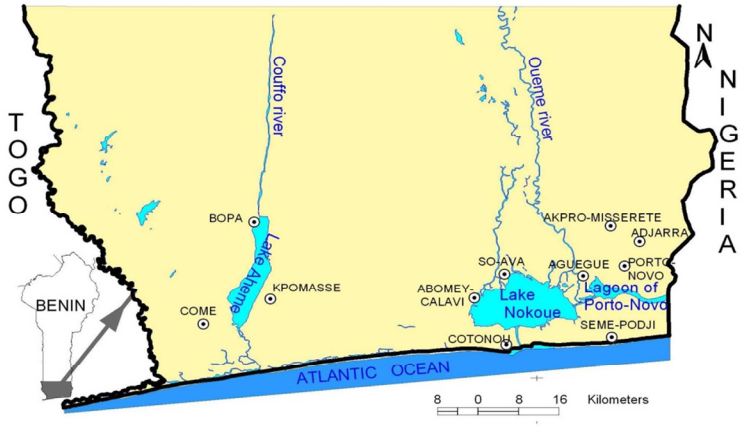

Fig. 2.1 Map of Benin showing the fishing areas and the capital of the municipalities included in the study (Adapted from the topographic background of the national geographic institute, 1992).

Questionnaires design and field data collection

Questionnaires were formulated for each group of stakeholders. The questionnaires were divided into four main parts. The first part dealt with demographic characteristics of the interviewee (name, age, gender, level of education, contact details). The second part was related to the stakeholder's shrimp handling practices. The third part investigated the interviewee's shrimp quality perceptions, and the fourth part focused on the relationship between the interviewee and his/her buyer, i.e. the next stakeholder along the chain. Questionnaires for freezing plant managers also included questions related to shrimp processing steps. The questionnaires consisted mostly of multiple answer questions which included the answer choice "other". The answer choice "other" was further specified by the respondents. The questionnaires were administrated using face-to-face interviews by three interviewers. The interviewers were agronomic engineers experienced in conducting surveys in rural areas. A training workshop was organized to get the interviewers familiarized with the objectives of the study, the content of the questionnaires, and the appropriate way to meet the respondents. The questionnaires were first tested in a pilot survey that included 17 fishermen, 7 intermediate traders, 2 collectors and 1 shrimp freezing plant manager. Then, the questionnaires were adjusted when needed, and used for the formal survey.

Data were collected in the villages of selected municipalities from February to April 2011. In each village, interviews with randomly selected informants were conducted in their local language. The response rates were 99.1% for fishermen (n=325) and 100% for intermediate traders (n=128), collectors (n=12), or shrimp plants managers (n=3).

Data analysis

Raw data were recorded in Microsoft Excel and descriptive statistics were calculated. Data related to fishing duration and shrimp storage periods by the fishermen and the intermediate traders were subjected to analysis of

variance using SASv9.2 software, followed by a Tukey's multiple comparison test. Significance was accepted at a *P* value of <0.05.

Results

The structure of the Beninese export-oriented shrimp chain

The socio-demographic profile of the respondents is presented in Table 2.1. All shrimp fishers respondents were male while almost all intermediate traders (97.7%) were female. Most of the respondents were between 41 and 50 years old. Most of shrimp fishers and intermediate traders did not receive any formal education, while shrimp plant managers had at least secondary school level.

Table 2.1

Socio-demographic profile of the respondents (in %)

	Fishermen (*n*=325)	Intermediate traders 1 and 2 (*n*=128)	Collectors (*n*=12)	Plant managers (*n*=3)
Gender				
Male	100	2.3	75	100
Female	0	97.7	25	0
Age (years)				
<20	0.6	0	0	0
21 – 30	22.8	10.1	8.3	0
31 – 40	22.8	26.6	33.3	33.3
41 – 50	26.1	43.0	33.3	66.7
51 – 60	20.6	16.4	25.0	0
>60	7.1	3.9	0	0
Educational level				
No schooling	63.4	87.5	33.3	0
Primary school	24.0	9.4	41.7	0
Junior secondary school	10.5	2.3	16.7	0
Senior secondary school	2.1	0.8	8.3	33.3
University	0	0	0	66.7

Diverse relationships exist among stakeholders involved in the Beninese shrimp chain (Fig. 2.2). After fishing, some fishermen (33% of respondents) sell shrimps to their wives, the intermediate traders 1. Most of these women (77% of respondents) sell their product to women or men specializing in shrimp precollection, the intermediate traders 2, who also buy shrimps directly from 58% of fishermen interviewed. The difference between the intermediate traders 1 and intermediate traders 2 is that the former buy shrimps only from their husbands, while the latter buy their products from various fishermen and intermediate traders 1. Special relations may exist between the fishermen and the intermediate traders 2. For instance, intermediate traders 2 provide fishing tackles and loans in advance to 15% and 11% of fishermen, respectively. Some fishermen (5% of respondents) sell their products to intermediate traders 2, solely because of a family relationship. In addition to these types of relationships, the intermediate traders 2 give food and drink to fishermen selling them shrimps in order to ensure their loyalty. The intermediate traders 2 in turn sell shrimps to the collectors, who can also be supplied directly by the fishermen or their wives (intermediate traders 1). However, there is a conflict of interest between the fishermen or their wives and intermediates traders 2 who believe that all the shrimps should be channeled through them to collectors.

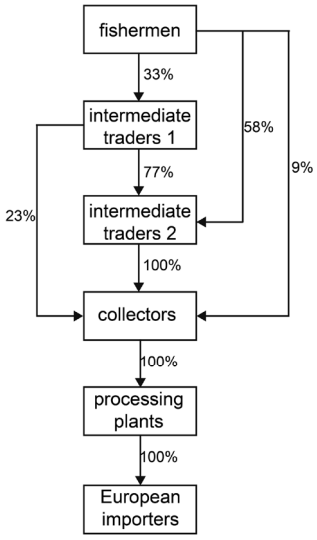

Fig. 2.2 The export-oriented shrimp chain in Benin. The percentage (%) indicates the proportion of stakeholders using the specified route.

Thirty-two percent of intermediate traders 1 and 2 asserted having particular relations with the collectors, who either motivate them in the beginning of the shrimp season by organizing a party or give them some money in advance. The collectors transport shrimps directly to the plants where they are processed and exported to Europe. In order to gain the confidence of collectors, plants' owners take various incentive measures. For example, the plant can put its means of transport at the collectors' disposal and reward the best suppliers at the end of the shrimp season.

Shrimp handling and processing

Shrimp fishing in Benin is an activity held by the communities settled on or near water bodies, which pass it on from generation to generation. More than 97% of the interviewed fishermen confirmed having learnt shrimp fishing from their relatives. Fishing generally takes place at night or early in the morning (4:00 to 6:00 a.m.). The fishing season varies, but it generally runs from December to September. Table 2.2 shows the fishing nets, shrimps holding containers, and the storage methods used by the fishermen. The most widely used net is the drift net. However, fishing nets with fine mesh (<20 mm) called *Medokpokonou* and *Gbagbaloulou*, which were prohibited (Anonymous, 1998), are still used in Lake Nokoue and Lake Aheme, respectively.

The captured shrimps are often poured directly in a wooden boat (62.5% of respondents). Refrigerated storage of shrimps by the fishermen is not a common practice in Benin. Only 3.4% of the interviewed fishermen store their shrimps on ice. The shrimps are stored at ambient temperature (28°C±1°C) by most of the fishermen (94.1% of respondents) or, sometimes, kept in a plastic basket containing the lake or lagoon water with an average temperature of 23.5°C±0.5°C (2.5% of respondents). There is a significant difference (P<0.05) between the average fishing duration of the various types of fishing nets. The fishing duration of fishermen using drift nets was 5.6±1.3 h (n=160). This time was approximately two times higher than the time spent by the fishermen who use bow nets (n=32), *Medokpokonou* (n=82), or cast nets (n=6). The fishing duration using gill nets and *Gbagbaloulou* were 3.6±0.9 h (n=39) and 3.2±1.2 h (n=6), respectively. During shrimp fishing with drift nets, 3 or 4 successive captures are realized and poured into the same container. About 4 h elapse between the first capture and the last one. After fishing, fishermen who use ice keep the shrimps for 4.1±3.7 h (n=11). This

holding time was significantly longer (P<0.05) than the holding time by fishermen who store shrimp in lake or lagoon water (1.4±1.2 h) (n=8) or at ambient temperature (1.4±0.6 h) (n=306).

Table 2.2

Fishing nets, shrimps holding container and storage methods used by fishermen

	Variant	Frequency (%) of informants[3] using the variant
Fishing net	drift net	49.2
	Medokpokonou[1]	25.2
	gill net	12.0
	bow net	9.8
	Gbagbaloulou[2]	1.9
	cast net	1.9
Holding container	Boat	62.5
	plastic basket	20.6
	wooden basket	13.8
	polystyrene bags	2.2
	Coolbox	0.9
Storage method	ambient temperature (28°C)	94.1
	Ice	3.4
	lake or lagoon water (23.5°C)	2.5

[1]*Medokpokonou* means literally "only one person laughs". It is the name given to a net with fine mesh capable of capturing even the small shrimps and other fishery products; [2]*Gbagbaloulou* is also a local name given to a tapered net with fine mesh; [3]Total number of fishermen is 325.

The holding time between the end of fishing and delivery allows fishermen to get in touch with their customers and, also, to separate shrimps from other aquatic species captured.

Shrimp precollection is conducted by intermediate traders 1 and 2 who receive shrimps at home or on shore. Baskets are the most used materials for the precollection (61.7% of the respondents). During the precollection, shrimps are stored on ice by 25% of the intermediate traders. Most of the intermediate traders (60.9%) just wash the shrimps using water from the lake, lagoon, well, or pump, and keep their products at ambient temperature (28°C±1°C) in the holding container until their delivery. The remaining (14.1%) store washed shrimps in water (23.5°C±0.5°C) until the time of sale. The shrimp holding time by intermediate traders that store shrimps at ambient temperature was 1.5±0.8 h (n=78). This holding time was significantly shorter time (P<0.05) than the holding time by intermediate traders who store shrimp in ice (3.1±2.0 h) (n=32) or in lake or lagoon water (2.9±1.7 h) (n=18).

Like shrimp precollection, shrimp collection takes place in an open environment where intermediate traders and some fishermen deliver their products to collectors. All the collectors use iceboxes in which they store collected shrimps on ice. The ice used is generally provided by shrimp processing plants. However, collectors are sometimes supplied with ice at the local market. The water used to make ice is from tap water and meets the standards mentioned in the decree No 2001-094 (Anonymous, 2001). After the collection which lasts on average 4.7±1.9 h (n=12), shrimps are transported by taxi. In the villages with difficult access, part of the transport of shrimps is carried out on motorcycle or by boat. The final destination of shrimps is one of the three shrimp processing plants in Cotonou (economic capital of Benin). The average duration of transport is 2.1±1.2 h (n=12).

Upon receipt of the shrimps, the processors carry out a sensory examination based on the color, consistency, smell and the presence of foreign bodies including sticks and meshes. The temperature in the center of the mass of shrimps is then measured using a probe thermometer. This temperature should be lower than 7°C according to the plant managers interviewed. However, when the temperature is between 7°C and 10°C, the shrimps are accepted but with a rapid lowering of temperature by addition of ice. Shrimps are rejected when their temperature is higher than 10°C. The rejected shrimps are smoked or fried and sold in Benin and in the subregion, mainly in Nigeria according to the plant managers interviewed. Sometimes shrimps of large size

without any physical damage and showing no sign of spoilage are just washed, kept on ice, and exported to European countries, namely, Belgium, France and Spain. However, shrimps are often subjected to different processing steps (Fig. 2.3) prior to export.

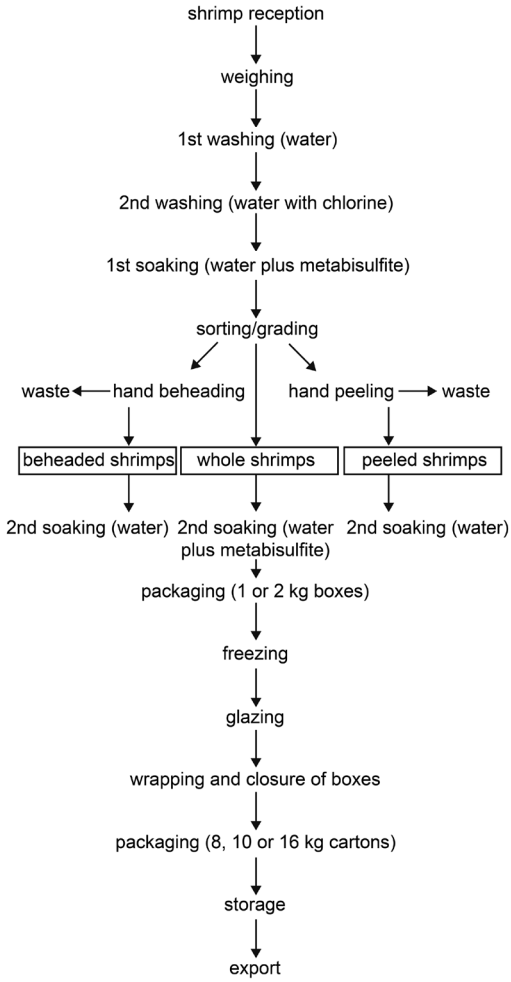

Fig. 2.3 Flow chart of plant-level shrimp processing for exportation purposes.

Received shrimps are weighed and washed with tap water at a temperature of about 5°C to get rid of sand and foreign bodies. Shrimps are washed a second time in water of about 5°C containing active chlorine at 0.2-0.5 ppm for 1 min to kill some microorganisms. Afterwards, they are soaked in a solution of 1% metabisulfite for 1-3 min at about 5°C to prevent melanosis (blackspot) development. Shrimps are then sorted, graded and distributed in three processing lines: beheaded shrimps, peeled shrimps and entire shrimps. The entire shrimps undergo a second soaking in a solution of 0.25% metabisulfite for 1-2 min at about 5°C, whereas the beheaded and peeled shrimps are soaked in simple water for 3 min at about 5°C. The shrimps are then packed in boxes of 1 kg or 2 kg, frozen at -40°C, and glazed at about 5°C. To protect the shrimps, they are wrapped using

sterile shrink films. The boxes are closed and packed in cartons of 8, 10 or 16 kg. The cartons are stored in a freezing room (about -20°C) and then exported to Europe.

Stakeholders' quality perception

The different groups of stakeholders do not have the same perception of shrimp quality (Table 2.3). The majority of fishermen, intermediate traders 1 and 2, and collectors consider shrimp size and texture their first two priority quality attributes, whereas at the plant level, plant managers consider the appearance (freshness) and the texture of shrimps their first two priority quality attributes.

As far as quality criteria are concerned, all stakeholders prefer shrimp that is large (at least 15 g per piece of shrimp), firm, and without any physical damage (whole shrimps without injury). Shrimp freshness is evaluated based on its carapace, which should be shiny. Also fresh shrimp should keep their natural (grey) color especially in the head where a red color is perceived as a sign of spoilage, and should not produce any off-odor. The decision on a batch of shrimp which does not meet the quality criteria depends on the stakeholder who wants to buy the product. The intermediate traders 2 accept the product, but may reduce the price; the collectors sort out the shrimp that complies with their criteria and reject the remaider; and the processors reject the entire batch.

Table 2.3

Stakeholders' first two priority quality attributes

	% whose priority quality attributes are[a]:					
Stakeholders	1, 2	1, 3	1, 4	2, 1	2, 3	5, 2
Fishermen (*n*=325)	72.3	26.1	0	0.9	0.6	0
Intermediate traders 1 and 2 (*n*=128)	68.0	22.6	7.8	1.6	0	0
Collectors (*n*=12)	75.0	8.3	8.3	8.3	0	0
Plant managers (*n*=3)	0	0	0	0	0	100

[a]Quality attribute 1=size; 2=texture; 3=color; 4=physical damage; 5=appearance.

Discussion

Shrimp chain structure

The structure of the shrimp chain in Benin shows that there are many specific relations between the stakeholders. According to Harland (1996), relationships between stakeholders represent valuable bridges as they give one stakeholder access to the resources of another. According to Trienekens et al. (2012), the acceptance of a product by the stakeholders of a supply chain depends on a combination of price, quality and safety, and associated guarantees. However, in the Beninese shrimp supply chain, some stakeholders may accept the shrimps only on the basis of the relationships existing between stakeholders. For instance, some wives buy their husband's shrimps at normal price whatever their quality because according to the customs, a woman should not oppose her husband. This asymmetric relation, i.e. unbalanced power between stakeholders (Zigger and Trienekens, 1999), may affect shrimp quality as the fishermen may not be motivated to improve the quality of their products.

The intermediate traders 2 accept all the shrimps from the fishermen or the intermediate traders 1 because they smoke the shrimps of marginal quality, which they sell at the local and subregional markets. However, the quality of shrimps intended for export can also be affected and shrimps will not meet the quality criteria stated in the inspection manual procedures of Beninese fishery directorate (unpublished document). Deficiencies related to shrimp quality at this stage of the chain can affect the end product. In fact, the quality and safety of the end product in a food supply chain depend on the role played by all members of the supply chain (Trienekens et al., 2012).

Shrimp handling

Many shortcomings related to shrimp quality were observed during the survey. The captured shrimps are dumped in the wooden boats where different catches are mixed. This practice does not meet the requirements of European Commission Regulation No 853/2004 (EC, 2004), which states that each successive catch must be kept separated. Moreover, the cold chain is not well maintained. Shrimps are stored at ambient temperature (28°C) during the fishing and the precollection before their delivery to the collectors who finally put them on ice. This practice is in violation of the requirements of the Codex Alimentarius (CAC, 2003) and of the European Regulation (EC, 2004), which stipulate that chilling of fishery products should start as soon as possible. In fact, shrimps are extremely sensitive to deterioration (Mendes et al., 2002). Unlike other crustaceans (crabs, lobsters), which can be kept alive until processing, shrimps die soon after being caught, and they are often contaminated with bacteria from their endogenous microflora as well as from the mud trawled up with them (Adams and Moss, 2000). Therefore, the storage temperature is crucial (Cyprian et al., 2008; Matches, 1982). For instance, as reported by Mendes et al. (2002), sensory analysis on fresh samples showed that raw chilled shrimps reached the limit of acceptability (50% rejection) after four days in ice, whereas 100% of the samples stored at room temperature (22°C) were rejected after 24 h.

In addition, the quality of lake and lagoon water used to wash shrimps is not in agreement with the requirements of European Commission Regulation No 853/2004 (EC, 2004). This nonpotable water may be a source of contamination. In fact, the microbial status of seafood after catch is closely related to environmental conditions and the microbiological quality of the water (Feldhusen, 2000). Also, shrimp collection in an unprotected environment is in violation of the European Commission Regulation No 853/2004 (EC, 2004), which states that fishery products must be kept in a protected environment.

Our survey showed that in Benin, shrimps are not handled properly and therefore may pose a potential risk to public health because improper handling practices may cause food contamination with pathogens and consequently foodborne disease (Huss et al., 2000; Plahar et al., 1999; Todd et al., 2010). Stakeholders involved in the Beninese shrimp sector should meet one of the key principles of the European regulations on the hygiene of foodstuffs (EC, 2004), which stipulates that it is necessary to ensure food safety throughout the food chain, starting with the primary product. This is of great importance given that food safety starts at primary production (Havelaar et al., 2010) and further processing can never restore the poor quality of shrimps improperly handled between the harvesting and the processing periods (Antony et al., 2002).

To ensure shrimp quality and safety there is a need to train the fishermen in Good Fishing Practice, and shrimp handlers, on safe food handling practices. However, it is known that food hygiene training will only be effective if the resources and systems are in place to encourage food handlers to implement good practices (Clayton et al., 2002; Seaman and Eves, 2010). Therefore, other measures such as building adequate facilities at the landing sites for shrimp collection and providing incentives for fishermen and shrimp handlers to improve hygiene, are needed. In addition, the use of ice by upstream stakeholders (fishermen and intermediate traders) should be promoted.

Shrimp quality perception

The upstream stakeholders in the chain give priority to shrimp size because they can make more money with shrimps that weigh more. There is concordance between our results and the results of Omemu and Aderoju (2008) who concluded from a survey conducted in Benin's neighboring country (Nigeria) that vendors considered the volume and the price more important than the freshness and cleanliness when buying food to be cooked or vended. The fact that non fresh shrimps can be smoked or fried and sold in Benin or in the subregion also explains why the upstream stakeholders do not give priority to shrimp freshness. The shrimp plant managers on the other hand give priority to shrimps freshness because they must ensure the quality of the exported products. In fact, products exported to Europe are regularly inspected on the market or at the border and

noncompliant products are rejected. Therefore, there is a need to inform upstream stakeholders about the importance of shrimp freshness and incentives may be developed to reach this goal.

In conclusion, this study shows that the conditions under which shrimps are handled in Benin are not in agreement with the requirements of European Regulations. The steps prior to shrimp processing at the plants, namely fishing, shrimp precollection and collection, are the critical steps for shrimp quality and safety in Benin because of temperature abuse and inappropriate hygienic conditions. To fulfill the requirements of European Regulations, joint efforts should be made. Because of the importance of foreign currency to its economy, Benin should provide training in Good Fishing Practice and safe food handling practices and develop incentives to improve shrimp quality and safety. The compliance with good practices should also be evaluated through monitoring. Moreover, it is important to ensure that the relationship between stakeholders, for example family relationship, do not pose a threat to shrimp quality and safety. This is one the challenging factors to be taken into account in the strategies to improve shrimp quality and safety. The importing countries might provide technical and managerial assistance for exporting countries with limited resources to implement the food safety requirements. A ban on products from exporting countries will certainly protect the health of their European Union consumers, but may also prevent these consumers from consuming some foods they have particular interest in, for instance, because of their organoleptic qualities. Our study highlights how a chain-wide survey can reveal technical and social aspects of relevance for better management of food safety and quality along the chain. Also, it provides data on time and temperature regimes along the chain, which can be used to evaluate relevant scenarios to determine growth of pathogenic organisms and spoilers in future studies.

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3

Bacterial concentration and diversity in fresh tropical shrimps (*Penaeus notialis*) and the surrounding brackish waters and sediment

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Abstract

This study aimed at determining bacterial concentration and diversity in fresh tropical shrimps (Penaeus notialis*) and their surrounding brackish waters and sediment. Freshly caught shrimp, water and sediment samples were collected in Lakes Nokoue and Aheme in Benin (West Africa) during two periods with different water salinity and temperature and fishing activity: June (12.9-13.8‰ salinity, 26.8-27.4°C, active fishing period) and August (2.5-2.9‰ salinity, 24.2-24.8°C, less active fishing period). We used culture dependent and culture independent methods for microbiota analysis. During both sampling periods, total mesophilic aerobic counts in shrimp samples ranged between 4.4 and 5.9 log CFU/g and were significantly higher than in water or sediment samples. In contrast, bacterial diversity was higher in sediment or water than in shrimps. The dominant phyla were* Firmicutes *and* Proteobacteria *in shrimps,* Firmicutes, Proteobacteria, *and* Actinobacteria *in water, and* Proteobacteria *and* Chloroflexi *in sediment. At species level, distinct bacterial communities were associated with sediment, water and shrimps sampled at the same site the same day. The study suggests that the bacterial community of tropical brackish water shrimps cannot be predicted from the microbiota of their aquatic environment. Thus, monitoring of microbiological quality of aquatic environments might not reflect shrimp microbiological quality.*

Introduction

Tropical brackish waters are endowed with highly diversified aquatic resources, which play a vital role in providing animal protein and income for the population (Adite and Van Thielen, 1995; Villanueva et al., 2006). In Benin, West Africa, shrimps (*Penaeus* spp.) are one of the fishery products caught from brackish waters, processed and sold in the local and regional markets, and also exported to Europe as raw frozen shrimp (Dabadé et al., 2014). Ensuring shrimp quality and safety for export is a big concern to shrimp stakeholders in Benin. For example in 2002, the Rapid Alert System for Food and Feed from Spain notified too high counts of *Enterobacteriaceae* (>10^3 CFU/g) and aerobic mesophiles (>10^6 CFU/g) in frozen shrimps from Benin and as a result, they could not be distributed (http://ec.europa.eu/food/food/rapidalert/index_en.htm). The type and concentration of bacteria associated with a frozen product depend not only on the conditions under which the product is handled and stored but also on the initial product quality (Laplace-Builhé et al., 1993). Shrimps die soon after being caught and they can be contaminated with bacteria from their endogenous microbiota as well as from their environment (Adams and Moss, 2000).

The documented microbiota of fresh caught shrimps varies according to shrimp species and origins. For example, Vanderzant et al. (1971) found that coryneform bacteria were the dominant microorganisms in pond-reared shrimps (*Penaeus aztecus*) from the West Galveston Bay (USA). *Flavobacterium* and *Planococcus* were found to be the most dominant microorganisms associated with *Penaeus* shrimps harvested from Florida's coasts (USA) (Alvarez, 1983). *Moraxella, Micrococcus* and *Pseudomonas* were the dominant microorganisms in fresh shrimps (*Penaeus merguiensis*) harvested in Pakistan tropical waters (Shamshad et al., 1990). Using culture-independent methods, Liu et al. (2011) showed that *Vibrio* species were the most abundant microorganisms in the intestinal tract of Chinese shrimps (*Fenneropenaeus chinensis*). Chaiyapechara et al. (2012) found differences in the microbiota of the intestinal tract of shrimps (*P. mondon*) reared in Thailand, but the dominant microorganisms were *Vibrio, Photobacterium,* or *Aeromonas*.

In Benin, to check for shrimp contamination, microbiological analysis is performed mainly on processed shrimps (FVO, 2003). Thus, little is known about the diversity and concentration of bacteria associated with freshly caught shrimps. In this study, we aimed at determining bacterial diversity and concentration in shrimps (*P. notialis*) caught from tropical brackish waters using a combination of culture-dependent and culture-independent approaches. The relationship between the microbiota of the shrimps and that of the surrounding waters and sediment was also investigated. Knowledge of the types of bacteria associated with shrimp and their initial concentrations is of importance to develop strategies and tools for a better shrimp quality and safety management.

Materials and methods

Field sites and sample collection

The main shrimp fishing areas in Benin, i.e. Lake Nokoue and Lake Aheme (Dabadé et al., 2014) were targeted. Lake Nokoue (6°20'-6°30'N, 2°20'-2°35'E) has a surface area of 150 km^2 and a depth of 1-3 m (Gadel and Texier, 1986; Villanueva et al., 2006). Lake Aheme (6°20'-6°40'N, 1°55'-2°E) has a surface area of 85 km^2 and a depth of 1-2.2 m (Maslin and Bouvet, 1986; Maslin and Pattee, 1989). Both lakes are connected with the Atlantic Ocean as well as to rivers (fresh water) from the North (Fig. S3.1). The salinity of the lakes varies from close to 0‰ during the flood season of the rivers up to 30‰ when the rivers recede (Lalèyè et al., 2003; Maslin and Bouvet, 1986). Water, sediment and shrimps were collected from three sites in Lake Nokoue and two sites in Lake Aheme in August 2011 (Fig. S3.1). In June 2012, two of the three 2011 sampling sites in Lake Nokoue (N1 and N3, Fig. S3.1) and one of the two 2011 sites in Lake Aheme (A1, Fig. S3.1) were again sampled. Four samples of each water, sediment and shrimp were collected per site. Shrimps were collected directly from the fishing nets using sterile hand gloves and put in sterile polyethylene bags. The gloves were changed between samples. Water samples were collected at 50 cm below the water surface and the sediments were collected at

their surface. The water parameters measured included salinity, pH and temperature (Table 3.1). Collected samples were immediately cooled (ice) and transported to the laboratory to arrive within 2 h.

Table 3.1
Parameters of lake waters during sampling in August and June (mean ± standard deviation)

Parameters	August (2011)		June (2012)	
	Lake Nokoue (*n*=12)	Lake Aheme (*n*=8)	Lake Nokoue (*n*=8)	Lake Aheme (*n*=4)
Salinity (‰)	2.5±0.5	2.9±0.6	12.9±0.1	13.8±0.5
pH	7.6±0.2	7.7±0.1	7.7±0.2	7.8±0
Temperature (ºC)	24.2±0.4	24.8±0.3	26.8±0.3	27.0±0.4

Enumeration and isolation of microorganisms

Whole shrimps (2-3 individuals per sample) were aseptically cut into small pieces. Cut shrimps or sediment samples of 25 g each were transferred aseptically to stomacher bags and diluted 10 times in physiological saline peptone solution (0.85% NaCl (SIGMA), 0.1% peptone (OXOID)). The mixture was homogenized for 60 s using a stomacher (Seward Laboratory Stomacher 400, England) to obtain the primary dilution from which appropriate decimal dilutions were prepared. For the water samples, decimal dilutions were prepared directly from the samples.

Aerobic mesophilic plate counts were enumerated on plate count agar (PCA, OXOID) incubated at 30˚C for 48 h and were reported as total viable count (TVC). *Enterobacteriaceae* were enumerated on pour plates of violet red bile glucose (VRBG, OXOID) overlaid with the same medium, incubated at 37ºC for 24 h. Oxidase and glucose fermentation tests were carried out on presumptive colonies of *Enterobacteriaceae*. *Pseudomonas* spp. were enumerated on spread plates of *Pseudomonas* agar base (OXOID) supplemented with cetrimide, fucidin, and cephaloridine (CFC) (OXOID), incubated at 25ºC for 48 h. Oxidase test was performed on their presumptive colonies. *Vibrio* spp. were enumerated on spread plates of thiosulfate citrate bile salt sucrose agar (TCBS, OXOID) incubated at 30ºC for 72 h. H_2S-producing bacteria were enumerated on double layered plates of iron agar (IA) supplemented with 0.04% L-cysteine (SIGMA) as previously described (Gram et al., 1987). Plates were incubated at 25ºC for 72 h and black colonies were counted as H_2S-producing bacteria. To enumerate spore forming bacteria, 5 mL of the primary dilution of shrimp or sediment samples and 5 mL of water samples were heated in sterile tubes for 10 min at 80ºC to kill the vegetative cells. Appropriate decimal dilutions were made from the heated samples. Spore forming bacteria were enumerated on plate count agar (PCA, OXOID) with overlay of 1.5% agar and incubated at 30ºC for 48 h. Lactic acid bacteria (LAB) were enumerated on double-layered plates of de Man, Rogosa and Sharp agar (MRSA) (OXOID) incubated at 30ºC for 72 h. Gram stain and catalase test were performed to confirm LAB.

For each group of microorganisms, colonies were picked randomly from PCA and streaked onto tryptone soya agar (TSA) (OXOID) plates for purification. Purified colonies were stored at -80ºC in 30% glycerol for further analyses.

DNA extraction

Genomic DNA was extracted from sediment samples (10 g wet weight per sample) using UltraClean Mega Prep Soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) following the manufacturer's protocol. The UltraClean water DNA Isolation kit (0.22 μm filters) (Mo Bio Laboratories, Inc., Loker Ave West, Carlsbad, CA, USA) was used to extract genomic DNA from water samples. The samples were first filtered using 0.22 μm filters and the DNA extraction following the manufacturer's protocol. Microbial genomic DNA extraction from shrimp matrix was performed by a combination of low and high speed centrifugation protocol followed by DNA purification using DNeasy Tissue Kit (Qiagen, Westburg b.v., the Netherlands) as previously described (Hovda

et al., 2007; Rudi et al., 2004). DNA extraction from colonies randomly isolated from PCA was performed following the protocol described in the genomic DNA purification kit (Promega Corporation).

PCR and DGGE analysis

Genomic DNA isolated from sediment, water and shrimp samples was used as template to amplify the V6-V8 region of the conserved 16S rRNA gene of bacteria using the set of primers previously described (Nubel et al., 1996). PCR mixture of 50 μl was made by addition of 1 μl of DNA template and 0.4 μM of each primer to a ready-to-use solution PCR master mix (Promega Corporation). The amplification conditions were as follows: initial denaturation of double stranded DNA at 94ºC for 5 min, then 35 amplification cycles with denaturation at 94ºC for 20 s, primer annealing at 56ºC for 20 s, and extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min followed by cooling at 4ºC. DGGE was applied to the PCR products using the Dcode System apparatus (Bio-Rad) as previously described (Martin et al., 2007; Muyzer et al., 1993). The DGGE gels were silver-stained as previously described (Sanguinetti et al., 1994), dried overnight at 55ºC and digitized using a GS 800 calibrated Densitometer (BioRad).

The PCR conditions described above were also used to amplify the 16S rRNA gene of bacterial colonies (randomly selected from plate count agar) DNA extracts using 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') universal primers as previously described (Lima et al., 2012).

Clones libraries construction

Three clone libraries were constructed for shrimp samples: two clone libraries for shrimp samples collected from the same site (N1) in Lake Nokoue during August and June sampling, respectively, and one clone library for shrimp samples collected during June sampling from the site (A1) in Lake Aheme. To obtain insight into the relationship between the microbiota of shrimps and that of their surroundings, two clone libraries were constructed for water and sediment samples, respectively collected at the same site (N1) and the same day (June sampling) as shrimp samples.

Amplified 16S rRNA gene using 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') universal primers was purified and cloned in *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) as described by Lima et al. (2012). The expected size (approximately 1500 bp) of the insert in recombinant colonies was checked by PCR using T7 and Sp6 pGem-T-specific primers as previously described (Lima et al., 2012). At least 96 clones were randomly selected for each clone library. An exception was the clone library for the shrimp samples collected from Lake Nokoue in August where only 60 clones were selected due to an insufficient number of recombinant colonies.

Sequencing

Clones (480 in total) and the PCR products of the bacterial isolates (38) were sequenced (GATC Biotech) with the bacterial universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3').

DGGE gels analysis

Digitized DGGE gels were analyzed using Bionumerics software 7.1 (Applied Maths, Belgium). The DGGE gels were normalized and a band matching analysis was performed. Similarity and dendrogram of the DGGE profiles were created based on the Dice correlation coefficient and unweighted pair-group method average (UPGMA). To assess the structural diversity of microbial community, Shannon index (H') and Simpson index (1-D) were calculated as previously described (Thavamani et al., 2012).

$$H' = -\sum \left[p_i \times \ln(p_i) \right] \quad (3.1)$$

$$1 - D = 1 - \sum {p_i}^2 \quad (3.2)$$

In both equations, $p_i = {n_i}/{N}$ with n_i the height of the peak, which corresponds to the band intensity in the densitometric curves and, N the sum of all peak heights of the bands in the densitometric curves.

Sequences analysis

Chimeric sequences were removed from the clone libraries and bacterial isolate sequences using DECIPHER (Wright et al., 2012). The Ribosomal Database (RDP) Classifier version 2.6, a naive Bayesian classification method (Wang et al., 2007), was used to perform taxonomic classification of the clone libraries sequences at 80% confidence threshold. The open-source software MOTHUR (Schloss et al., 2009) version 1.33 was used to assign the sequences to operational taxonomic units (OTUs) based on 97% sequences similarity cutoff (0.03 distance threshold). Shannon index of diversity (*H'*) and Simpson index of diversity (1-*D*) were determined using MOTHUR. The percentage of coverage of the clone libraries was calculated using Eq. (3.3) (Bai et al., 2012; Bekele et al., 2011; Good, 1953).

$$C = \left[1 - (n / N) \right] \times 100 \quad (3.3)$$

where C is the library coverage (in percentage), n is the number of singletons and N is the total number of sequences.

The representative sequence of each OTU was compared against the GenBank database using the basic local alignment search tool (BLAST) (Altschul et al., 1990). Phylogenetic analyses were conducted using MEGA 5.1 (Tamura et al., 2011) for shrimp clone libraries. The neighbor-joining method based on distance estimates calculated by the Jukes-Cantor model was used to construct the tree, which was bootstrap resampled 1,000 times.

Statistical analysis

The difference in average levels of the groups of microorganisms enumerated was assessed using a Student's two-tailed t-test or one-way ANOVA (IMB SPSS Statistics 19.0) followed by Tukey's test as post hoc comparison of means. Significance was accepted at $P<0.05$.

Nucleotides sequences accession numbers

The sequences obtained in this study have been deposited in the GenBank under the accession numbers KP181616 - KP181650 (isolates) and KP181651 - KP182059 (clones).

Results

Enumeration of bacterial groups

Microbial concentrations in shrimp, water and sediment samples are presented in Fig. 3.1. Of the August shrimp samples, the average concentration of aerobic plate counts reported as total viable counts (TVC) varied between 4.4 and 5.4 log colonies forming unit (CFU)/g (Fig. 3.1A). The concentrations of *Pseudomonas* spp. and *Vibrio* spp. were similar and varied between 3.6 and 4.5 log CFU/g. The concentration of H_2S-producing bacteria ranged between 3.2 and 4.0 log CFU/g, and for *Enterobacteriaceae,* the concentration varied between 1.4 log CFU/g, in sample A1-Aug and 3.2 log CFU/g, in sample N3-Aug. The average concentration of the different groups of microorganisms was significantly higher in shrimp than in water (Fig. 3.1B) or sediment (Fig. 3.1C), except for spore forming bacteria whose concentration was significantly higher in sediment.

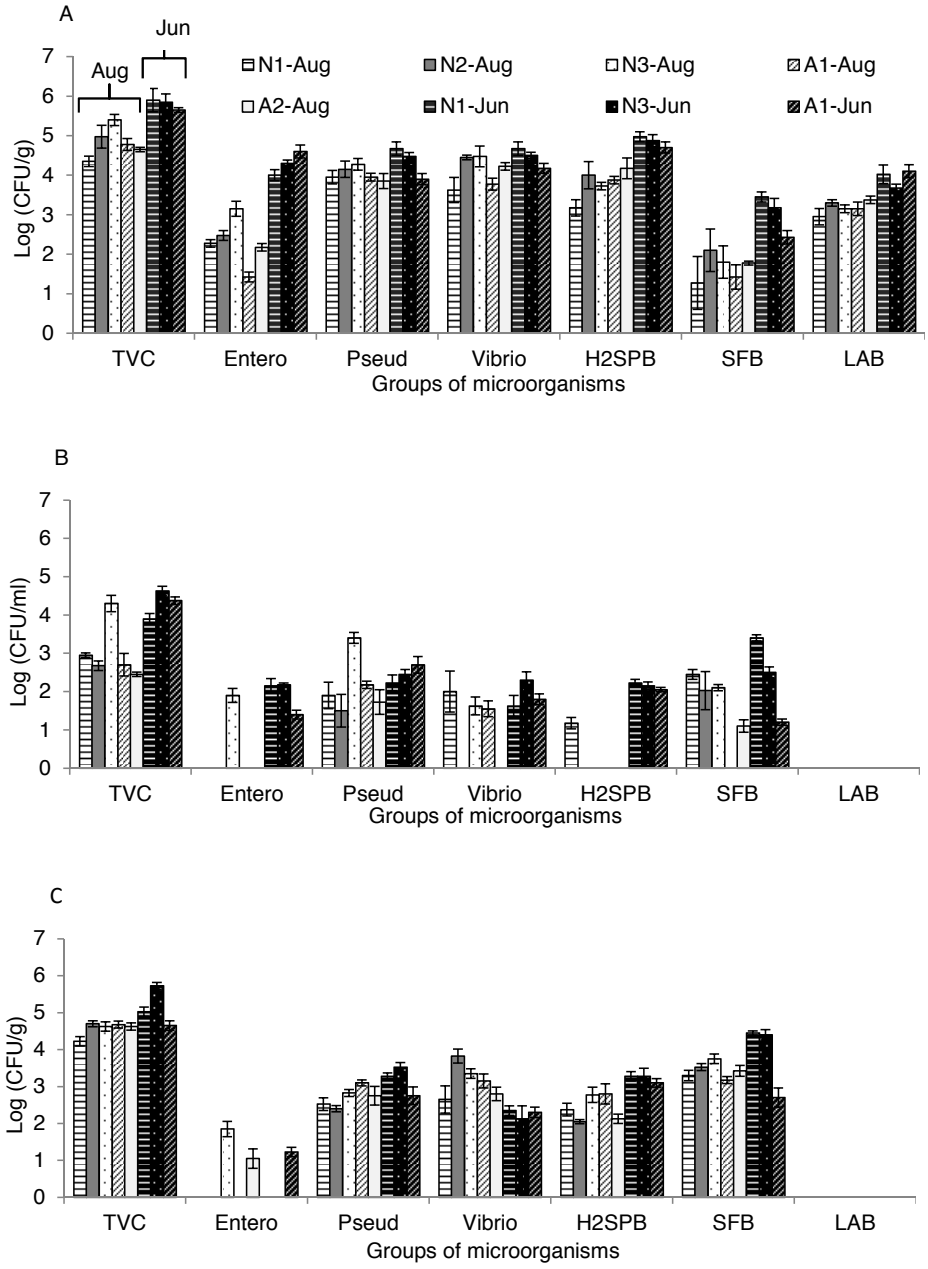

Fig. 3.1 Microbial concentrations of different groups of microorganisms enumerated in (A) shrimp samples, (B) water samples, and (C) sediment samples.

TVC = total viable counts, Entero = *Enterobacteriaceae*, Pseud = *Pseudomonas* spp., H2SPB = H_2S-producing bacteria, SFB= spore-forming bacteria, LAB = lactic acid bacteria. Ai-Aug = August samples from site i in Lake Aheme; Ni-Jun = June samples from site i in Lake Nokoue. Bars represent the standard deviation of four independent samples.

In general, the average concentration of the different groups of microorganisms in shrimp or water samples was higher in June than in August. The difference in the average concentration of the different groups of microorganisms in sediment during both periods was however less clear. During both periods, LAB average concentrations varied between 3 and 4 log CFU/g in shrimps to below detection limit (0 log CFU/ml in water samples and 1 log CFU/g in sediment samples) in water and sediment samples.

Bacterial community analysis by PCR-DGGE

Cluster analysis of DGGE profiles showed that shrimps were grouped separately from water and sediments (Fig. 3.2). DGGE profiles of shrimp bacteria sampled in June formed a separate cluster from those sampled in August, suggesting a seasonal effect on shrimp DGGE banding profiles. For water and sediments, the seasonal effect was not obvious since the DGGE profiles of the two sampling periods grouped partly together. It should be noted that some samples with distant geographic location such as shrimp samples collected in August from the third site in Lake Nokoue (ShN3-Aug) and the second site in Lake Aheme (ShA2-Aug) displayed high (92%) similarity (see Fig. 3.2).

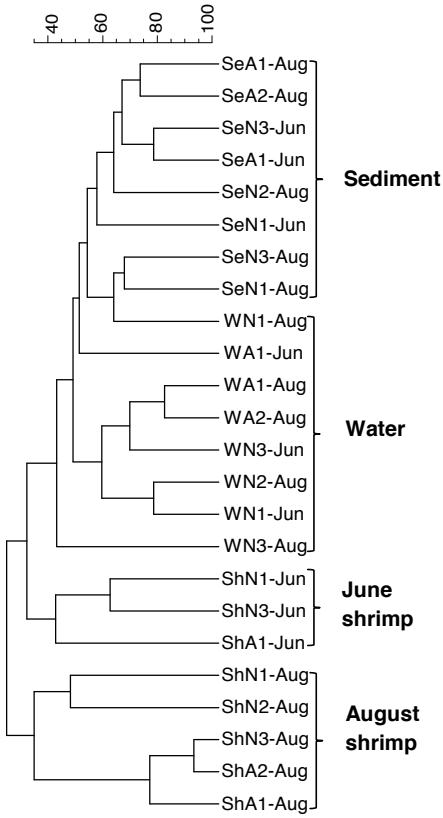

Fig. 3.2 Clustering analysis of DGGE profiles of sediment, water and shrimp sampled in June and August from Lake Nokoue and Lake Aheme.
Se = sediment; W = water; Sh = shrimp, Ni= site i in Lake Nokoue, Ai = site i in Lake Aheme, Jun = sampling period (June) and Aug = August.

Numerical analysis of the DGGE profiles based on the relative height intensity of each band indicated lower values of Shannon index of diversity (H') and Simpson index of diversity (1-D) in shrimp samples compared to water or sediment samples during both sampling periods (Fig. S3.2). The diversity indices were higher in June shrimps than in August shrimps, but were similar in water and sediment samples during both periods. The values of the diversity indices suggest that the bacterial community was less diverse in shrimp samples than in water or sediment samples.

Bacterial community analysis by clone libraries

After discarding the chimeric sequences, the number of sequences analyzed per sample ranged from 55 to 112 (Table 3.2). Based on RDP Classifier (80% confidence level), the number of phyla ranged from 1 (in August shrimp from lake Nokoue: ShN1-Aug) to 11 in the sediment sample (SeN1-Jun) (Fig. 3.3A). The proportion of unclassified bacteria was relatively high in the sediment sample (SeN1-Jun) (20% of the clone library, *n*=96), but low in other samples (0-2%).

Table 3.2

Number of sequences analyzed, richness and diversity indices of the clones libraries

Samples	Number of sequences analyzed	Number of OTUs (similarity≥97%)	Coverage index (C) (%)	Shannon index (H')[a]	Simpson index (1-D)[a]
ShN1-Jun	83	19	90.4	2.36 (2.13, 2.59)	0.87 (0.83, 0.91)
ShA1-Jun	112	20	89.3	1.92 (1.67, 2.17)	0.76 (0.70, 0.82)
ShN1-Aug	55	5	98.0	1.06 (0.85, 1.27)	0.60 (0.53, 0.67)
WN1-Jun	83	43	60.2	3.16 (2.86, 3.46)	0.91 (0.87, 0.96)
SeN1-Jun	96	77	32.6	4.25 (4.09, 4.41)	0.99 (0.98, 1.00)

[a]95% confidence interval lower bound and upper bound are shown in parentheses

ShN1-Jun, WN1-Jun, and SeN1-Jun = shrimp, water and sediment samples, respectively. These samples were collected at the same day during June sampling from site 1 in Lake Nokoue. ShN1-Aug = shrimp sample also collected from site 1 in Lake Nokoue, but during August sampling. ShA1-Jun = shrimp sample collected during June sampling from site 1 in Lake Aheme.

The most dominant phylum in shrimp samples from Lake Nokoue (both sampling periods) and the water sample from the same lake was *Firmicutes*. The phylum *Firmicutes* was however not recovered from the clone library of the sediment sample collected at the same site in the same lake. In contrast, the sediment sample was dominated by the phylum *Proteobacteria* (53% of the clone library, *n*=96). *Proteobacteria* predominated in shrimp sample from lake Aheme (ShA1-Jun) (82% of the clone library, *n*=112). *Proteobacteria* was the second most abundant phylum in water sample (WN1-Jun) (18% of the clone library, *n*=83) and the third most abundant in shrimp from Lake Nokoue sampled in June (ShN1-Jun) (10% of the clone library, *n*=83). However, *Proteobacteria* members were not evenly distributed within the classes of the phylum among the libraries (Fig. 3.3B). *Alphaproteobacteria* were the most abundant *Proteobacteria* in the water sample (47% of proteobacterial clones in the sample, *n*=15) while they represent the least abundant *Proteobacteria* in the sediment sample (10% of proteobacterial clones in the sample, *n*=51). *Gammaproteobacteria* were the most abundant *Proteobacteria* in the sediment sample (35% of proteobacterial clones in the sample, *n*=51) followed by *Deltaprotobacteria* (29%). However, *Deltaproteobacteria* were not recovered from the water sample. Shrimp samples were mainly dominated by *Gammaproteobacteria*.

With a 3% similarity cut-off (sequence similarity ≥97%), the sequences were assigned to operational taxonomic units (OTU) varying between 5 and 77 OTUs per sample (Table 3.2). The coverage index ranged between 33% (SeN1-Jun) and 98% (ShN1-Aug). As revealed by DGGE analysis, the Shannon index (H') and the Simpson index (1-D) of diversity were higher in the sediment and water sample than in shrimp samples. Within the shrimp samples, the indices of diversity were higher in the June samples than in the August samples, which is consistent with the DGGE results.

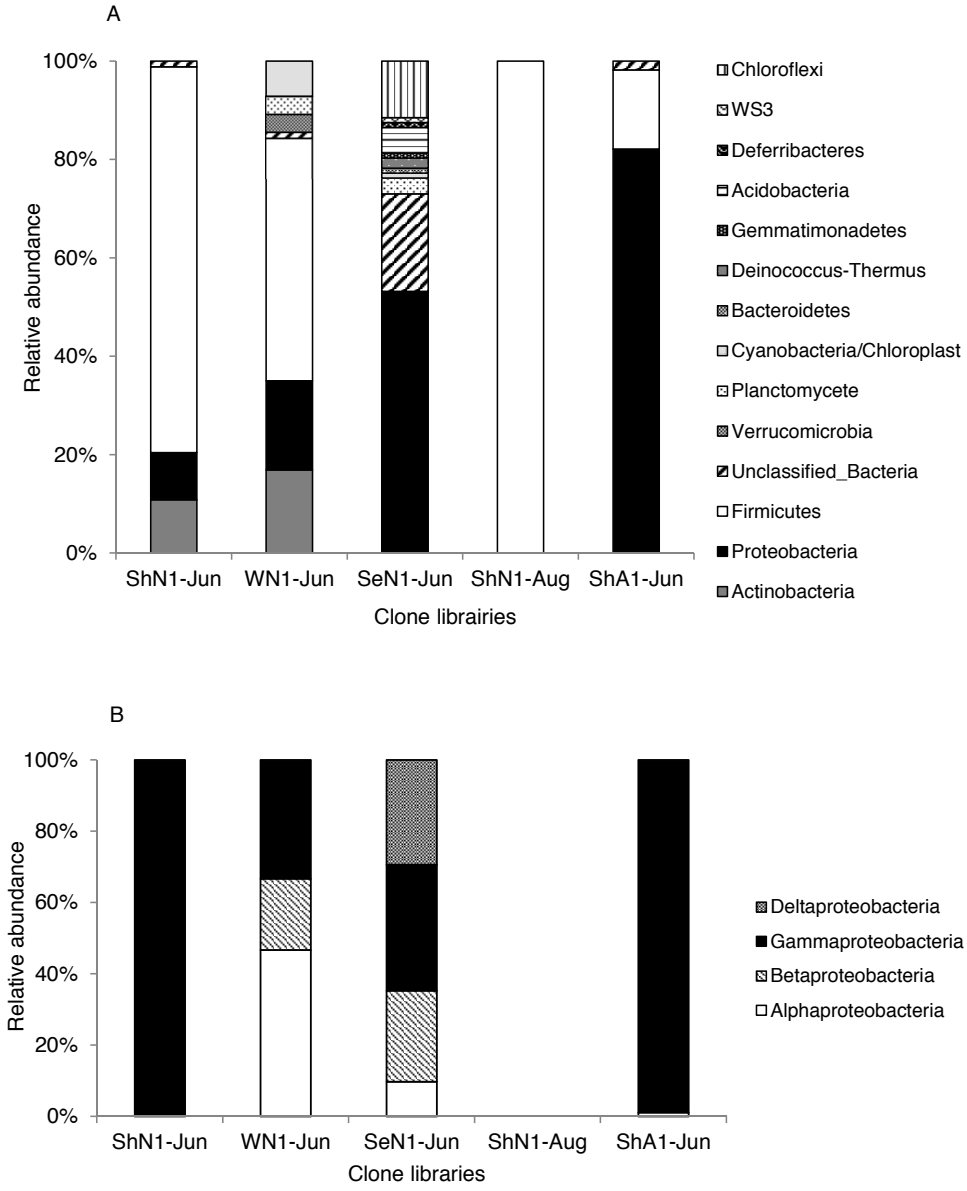

Fig. 3.3 Relative abundance of (A) the identified phyla in the clone libraries and (B) *Proteobacteria* classes within each clone library. For details about the samples names, see Table 3.2.

Phylogenetic affiliation of identified OTUs

By combining all the sequences from the 5 clone libraries, and discarding identical sequences using Mothur software, 424 unique sequences were obtained and assigned to 159 OTUs at 0.03 distance (sequence similarity ≥97%). Twenty two out of the 159 OTUs were considered as unclassified bacteria at 80% confidence threshold with the RDP Classifier. A BLAST search was performed on the representative sequence of each of the 137 remaining OTUs. Of the 137 OTUs, 38 (28%) exhibited <97% similarity with the sequences available in the GenBank, and therefore may represent novel species. For the OTUs showing ≥97% similarity with their closest relatives in the GenBank and belonging to shrimp clone libraries, a phylogenetic tree was constructed using the identities of these closest relatives (Fig. 3.4). The identities of OTUs belonging to the water and the sediment clone libraries are shown in Table S3.1.

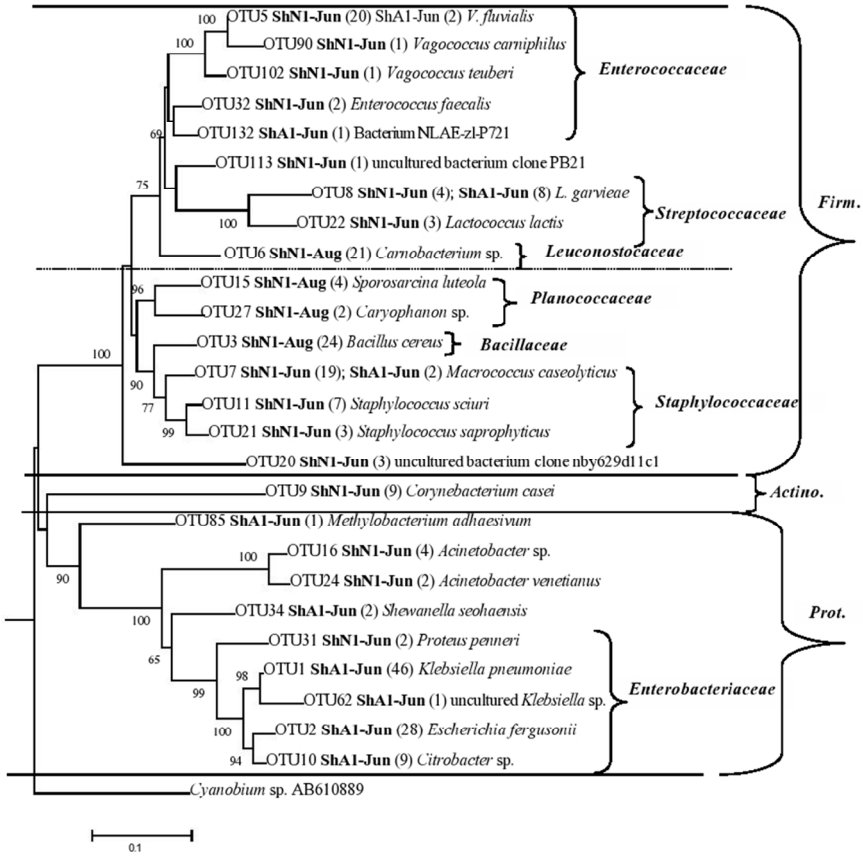

Fig. 3.4 Phylogenetic position of 16S rRNA sequences of clones from shrimps sampled in brackish waters in Benin (West Africa). The trees were generated using neighbor-joining method with 1,000 bootstrap replicates. Bootstrap values >50% are displayed on the branches. The tree was rooted with *Cyanobium* sp. followed by its GenBank accession number. The scale bar represents 10% sequence divergence. The tree was generated using the representatives of 16S rRNA sequences clustered at ≥97% similarity (operational taxonomic unit (OTU) defined at distance 0.03).The representative OTUs are followed by the samples (in boldface) from which they were recovered and the number of sequences in each OTU is in parenthesis after the samples names. Clones identities are the best NCBI BLAST matches. For details about the samples names, see Table 3.2. Abbreviation: *Firm.*= *Firmicutes*; *Actino.* = *Actinobacteria*; *Prot.* = *Proteobacteria*.

***OTUs assigned to* Firmicutes**

In shrimps clone libraries (Fig. 3.4), a cluster was represented by LAB namely members of the families *Enterococcaceae, Streptococcacaea*, and *Leuconostocaceae* (Fig. 3.4). OTUs from shrimps sampled in June were closely related to species belonging to the genera *Vagococcus* (OTUs 5, 90, and 102), *Enterococcus* (OTU32), and *Lactococcus* (OTUs 8 and 22), while OTU6 from shrimps sampled in August was closely related to a species of *Carnobacterium*. Three OTUs recovered from shrimps sampled in June were classified in the family *Staphylococcaceae*. One OTU (OTU3 from shrimps sampled in August) was closely related to *Bacillus cereus* and two others (OTUs 15 and 27) were closely related to members of the family *Planococcaceae*. In the water library (Table S3.1), LAB and *Staphylococcaceae* were not recovered. The dominant members of *Firmicutes* were *Exiguobacterium* species.

***OTUs assigned to* Proteobacteria**

Within the class *Gammaproteobacteria*, two OTUs (OTUs 16 and 24) belonging to *Acinetobacter* species were recovered. OTU34 from shrimps sampled in June in Lake Aheme fell into the family *Shewanellaceae*. The biggest cluster was represented by *Enterobacteriaceae*. Within the class *Alphaproteobacteria,* only one OTU (OTU85) from shrimps clone libraries, which is closely related to *Methylobacterium adhaesivum* was recovered. OTUs assigned to the phylum *Proteobacteria* recovered from water or sediment clones libraries clustered mainly with uncultured members of the phylum (Table S3.1).

OTUs assigned to other phyla

In shrimps clone libraries (Fig. 3.4), only the phylum *Actinobacteria* represented by *Corynebacterium casei* was recovered in addition to the phyla *Firmicutes* and *Proteobacteria*.

In water or sediment clones libraries recovered OTUs were mainly closely related to uncultured bacteria and clustered in different phyla such as *Actinobacteria, Planctomycetes, Acidobacteria, Chloroflexi, Verrumicrobia, Cyanobacteria, Gemmatimonadetes, Deinococcus-Thermus*.

Note that at 0.03 phylogenetic distance (species level), among OTUs from water, sediment and shrimps sampled at the same site the same day (June sampling). Only one OTU (OTU19, Table S3.1) closely related to uncultured *Xanthomonadales* (*Gammaproteobacteria*) was shared between the bacterial community of water and that of sediment. There was no overlap between OTUs from shrimps and those from sediment or water.

Bacterial isolates identities

The identities of bacterial isolates from plate counts agar (PCA) of the five cloned samples are presented in Table 3.3. The family *Staphylococcaceae* represented by *Staphylococcus* spp. and *Macrococcus caseolyticus*, also identified by cloning analysis, predominated in shrimp sampled in June. *Bacillus* spp. were identified in water and sediment sampled in June and in shrimp sampled in August. Other *Bacillales* such as *Exiguobacterium indicum* and *Kurthia gibsonii* were also identified in shrimp samples. LAB represented by species identified by cloning analysis, namely *Lactococcus garvieae* (OTU8), *Enterococcus faecalis* (OTU32) and other species such as *Pediococcus pentosaceus,* and *Enterococcus* spp. were identified in all shrimp samples. Isolates belonging to *Enterobacteriaceae* were identified as *Pantoea ananatis, Serratia marcescens, Escherichia fergusonii* in shrimp sampled in June from Lake Aheme, *Proteus vulgaris* in shrimp sampled in June from Lake Nokoue, and *Enterobacter* spp. in water sampled in June. Opportunistic pathogenic bacteria namely *Pseudomonas stutzeri* (Potvliege et al., 1987) and *Stenotrophomonas maltophilia* (Denton and Kerr, 1998) were also identified in the water sample.

Table 3.3
Identity of isolates randomly selected from plate count agar (reported as total viable counts) as revealed by 16S rRNA sequencing

R	Number of isolates	% Similarity and GenBank closest relatives	GenBank accession number
ShN1-Jun	(*n*=12)	99% *Staphylococcus sciuri*	KP181616
		100% *Staphylococcus sciuri*	KP181617
		97% *Staphylococcus sciuri*	KP181618
		99% *Staphylococcus sciuri*	KP181619
		100% *Staphylococcus xylosus*	KP181620
		98% *Staphylococcus* sp.	KP181621
		100% *Lactococcus garvieae*	KP181622
		98% *Kurthia gibsonii*	KP181623
		99% *Pediococcus pentosaceus*	KP181624
		100% *Proteus vulgaris*	KP181625
		99% *Macrococcus caseolyticus*	KP181626
		99% *Exiguobacterium indicum*	KP181627
WN1-Jun	(*n*=5)	100% *Enterobacter ludwigii*	KP181628
		100% *Enterobacter* sp.	KP181629
		100% *Pseudomonas stutzeri*	KP181630
		100% *Stenotrophomonas maltophilia*	KP181631
		100% *Bacillus pumilus*	KP181632
SeN1-Jun	(*n*=3)	100% *Bacillus licheniformis*	KP181633
		100% *Bacillus megaterium*	KP181634
		100% *Bacillus stratosphericus*	KP181635
ShN1-Aug	(*n*=6)	100% *Bacillus thuringiensis*	KP181636
		100% *Bacillus amyloliquefaciens*	KP181637
		99% *Enterococcus casseliflavus*	KP181638
		100% *Enterococcus faecalis*	KP181639
		100% *Enterococcus faecalis*	KP181640
		100% *Kurthia gibsonii*	KP181641
ShA1-Jun	(*n*=9)	99% *Staphylococcus nepalensis*	KP181642
		100% *Staphylococcus cohnii*	KP181643
		100% *Staphylococcus* sp.	KP181644
		99% *Macrococcus caseolyticus*	KP181645
		99% *Pantoea ananatis*	KP181646
		100% *Serratia marcescens*	KP181647
		100% *Kurthia gibsonii*	KP181648
		100% *Escherichia fergusonii*	KP181649
		95% *Enterococcus* sp.	KP181650

Discussion

The present study aimed to determine bacterial concentration and diversity in tropical brackish water shrimps and the relationship between the microbiota of the shrimps and that of the surrounding water and sediment.

Bacterial concentration

The initial total aerobic counts in shrimps obtained in this study is in agreement with earlier observations that in general, tropical shrimps carry high (5-6 log CFU/g) initial counts of bacteria (ICMSF, 2005). Therefore, one should realize that relatively high bacterial counts in fresh tropical shrimps may not necessary indicate that the product is of unacceptable quality. The initial concentration of H_2S-producing bacteria (4 log CFU/g) obtained in tropical shrimps from India (*Penaeus indicus*) (Jeyasekaran et al., 2006) is in the range of the initial concentration of H_2S-producing bacteria obtained in this study (3.2-5.0 log CFU/g). However, we found higher initial concentration of LAB (3.0-4.1 log CFU/g) in tropical brackish water shrimp than in the Indian shrimp (2

log CFU/g) (Jeyasekaran et al., 2006). The higher initial bacterial concentration in shrimp in comparison to water or sediments could be explained by a higher proportion of cultivable bacteria associated with shrimps. In fact, the majority of bacteria associated with environmental samples is not cultivable under laboratory conditions (Stevenson et al., 2004). Analyzing the microbial community of water and white shrimp (*Litopenaeus vannamei*), Johnson et al. (2008) also found that the microbial concentration in shrimp samples was higher than in water. Del'Duca et al. (2015) also reported higher bacterial concentration in tilapia (*Oreochromis niloticus*) than in water or sediment. During August sampling, the microbial concentration (TVC) in shrimps collected from the site 3 in Lake Nokoue (N3, Fig. S3.1) was significantly higher than the microbial concentration in shrimps collected from any other site (Fig. 3.1A). Similarly, *Enterobacteriaceae* concentration in shrimp, water and sediment from this site was significantly higher. A possible reason for this difference is that this site is located in the vicinity of the village of Ganvié where people live in pole-houses above the lake. Although found in shrimps LAB were not detected in water and sediment using both culture-dependent and culture-independent methods. This finding suggests that it is likely that LAB are indigenous of gastro-intestinal tract of shrimps. It has been reported that LAB constitute an important part of indigenous gut microbiota of shrimps (Maeda et al., 2014).

Bacterial diversity

The two molecular methods used in this study (PCR-DGGE and clone libraries) revealed that shrimps sampled in June had higher microbial diversity than in August. The salinity of the waters (ca. 12.9 ‰ in June and 2.5 ‰ in August) and their temperature (26.8°C in June and 24.2°C in August) are environmental parameters that could explain this difference. It has been documented that environmental factors can affect the composition of intestinal bacteria of invertebrates (Chaiyapechara et al., 2012; Hagi et al., 2004; Harris, 1993; Sullam et al., 2012). Variation in bacterial composition of shrimps sampled in the same period (June) was also found in this study. Chaiyapechara et al. (2012) also found differences in species composition among individual shrimps harvested from the same farm. This suggests that other factors than environmental factors might influence the composition of bacteria associated with shrimps. For instance, Rungrassamee et al. (2013) showed that there was difference in the bacterial population associated with shrimps (*Penaeus monodon*) at different developmental stages.

Our study showed a higher bacterial diversity in water or sediment samples than in shrimps. This result is in agreement with the report on water and shrimp microbial diversity by Johnson et al. (2008) who showed that water microbiota were more diverse than shrimp microbiota.

The phylum *Firmicutes* was the most abundant in shrimp samples from Lake Nokoue and the second most abundant phylum in shrimp samples from Lake Aheme. *Firmicutes* members have been reported to be associated with the intestinal tract of some shrimp species namely, *Fenneropenaeus chinensis* (Liu et al., 2011), *Rimicaris exoculata* (Durand et al., 2010), and *Penaeus monodon* (Chaiyapechara et al., 2012; Rungrassamee et al., 2014). Apart from Rungrassamee et al. (2014) who found *Firmicutes* to be dominant in only one of the six clone libraries that they analyzed, the abundance of *Firmicutes* associated with shrimps reported in literature is low in general. *Firmicutes* members were not even detected in the intestinal tract of *Penaeus merguiensis* (Oxley et al., 2002), *Litopenaeus vannamei* (Johnson et al., 2008), and *Marsupenaeus japonicus* (Liu et al., 2010). The most abundant genus of *Firmicutes* (*Fusibacter*) (Chaiyapechara et al., 2012; Rungrassamee et al., 2014) associated with black tiger shrimp *Penaeus monodon* was not recovered from any of *Penaeus notialis* samples in our study. LAB were the most abundant group of *Firmicutes* found in shrimp samples in the present work. LAB were mainly represented by *Lactococcus garvieae, Vagococcus fluvialis, Carnobacterium* spp., and *Enterococcus faecalis*. Jaffres et al. (2009) also found that *Carnobacterium* spp., *Vagococcus* spp., and *Enterococcus* spp. were the dominant bacterial strains of the spoilage microbiota of tropical cooked and peeled shrimps, *Penaeus vannamei*. Dalgaard et al. (2003) also isolated *Lactococcus garvieae* from spoilage microbiota of warm-water cooked and brined shrimps. *Carnobacterium maltaromaticum* was found to be responsible for

strong and rapid spoilage of cooked whole tropical shrimps (*Penaeus vannamei*) stored under modified atmosphere packaging (Mace et al., 2014).

The family *Staphylococcaceae* represented another member of *Firmicutes* recovered from shrimps sampled in June. The genus *Staphylococcus* has been detected in brown shrimps (*Crangnon crangnon*) (Broekaert et al., 2013) and in raw frozen black tiger shrimp imported into Denmark from Vietnam (Noor Uddin et al., 2013). The most common pathogenic species (*Staphylococococcus aureus*) was not detected in the present work. However, *Staphylococcocus sciuri* identified in our study has been reported in the same country (Benin) to cause a nosocomial bacteremia outbreak in hospitalized patients (Ahoyo et al., 2013). To our knowledge, this is the first time *Macrococcus caseolyticus* has been detected in shrimps (*Penaeus notialis*). It has been demonstrated that although *Macrococcus caseolyticus* is closely related to the genus *Staphyloccocus*, it lacks many of the virulence genes that are present in *Staphylococcocus aureus* (Baba et al., 2009). It is also known that the second closest relatives of *Macrococcus caseolyticus* are *Bacillus* spp. (Baba et al., 2009). Our finding is in agreement with these reports since a clade made of *Staphylococcus* spp., *Macrococcus caseolyticus,* and *Bacillus cereus* with 90% bootstrap value was obtained in our phylogenetic tree (Fig. 3.4).

Like in several previous studies on shrimp microbial diversity (Chaiyapechara et al., 2012; Johnson et al., 2008; Liu et al., 2011; Rungrassamee et al., 2014; Oxley et al., 2002), we found *Proteobacteria*, specifically members of the class *Gammaproteobacteria* to be the most abundant bacteria in our clone library for shrimp sampled in June in Lake Aheme (81% of the clones in the library, *n*=112). However, *Proteobacteria* were less abundant in the clone library for shrimp sampled in June in Lake Nokoue (10% of the clones in the library, *n*=83). Members of *Proteobacteria* namely *Klebsiella pneumoniae* (Choudhury and Kumar, 1998), *Acinetobacter* spp. (Broekaert et al., 2013; Heinsz, 1988; Liu et al., 2011) have been previously detected in shrimps. Our study identified for the first time *Shewanella seohaensis* in shrimps. This species was recently identified in tidal flat sediment samples in Korea as a novel species of the genus *Shewanella* (Yoon et al., 2012).

The phylum *Actinobacteria* (Fig. 3.4) was another important phylum detected in the present work from shrimps sampled in June in Lake Nokoue. Actinobacterial clones detected in the shrimp sample were closely related to *Corynebacterium casei*. This organism was isolated for the first time in cheese (Brennan et al., 2001). To our knowledge, this is the first time that *Corynebacterium casei* has been detected in penaeid shrimps.

The identification of some isolates randomly picked from plate counts agar (PCA) (Table 3.3) of shrimp samples revealed some bacteria such as *Lactococcus garvieae, Escherichia fergusonii, Proteus vulgaris,* and *Enterococcus faecalis* members of the family *Staphylococcaceae* (*Staphylococcus* spp. and *Macrococcus caseolyticus*), which were also detected in the clone libraries of shrimp samples. This identification based on culture-dependent approach showed that members of the family *Staphylococcaceae* (*Staphylococcus* spp. and *Macrococcus caseolyticus*) and some Gram-positive non spore-forming bacteria such as *Kurthia gibsonii,* and *Exiguobacterium indicum* are groups of cultivable bacteria that were not enumerated in this study.

Relationship between the microbiota of the shrimps and that of the surrounding water and sediment

In the present study, we found that at species level, distinct bacterial communities were associated with water, sediment and shrimps, sampled at the same site and day. Although limited numbers of clone libraries for water and sediment were used in this study, some previous studies have also reported different bacterial assemblages between water and sediment samples (Cole et al., 2013; Feng et al., 2009). In our study, one can argue that the relatively low percentage of coverage of sediment and water clone libraries at species level (0.03 phylogenetic distance) (Table 3.2) could justify the low overlap found between shrimp bacterial communities and that of their aquatic environment. However, even at family level (0.10 phylogenetic distance) where the coverage of the water clone library is 85% (data not shown), only one OTU, the family *Moraxellaceae*, is shared between shrimps and water samples. At order level (0.15 phylogenetic distance) where the coverage of the sediment clone library is 71% (data not shown), still no OTU is shared between shrimp and sediment samples. These findings

suggest that monitoring of the microbiological quality of aquatic environments might not be enough to get insight into the microbiological quality of shrimps.

In conclusion, this study combined culture-dependent and culture-independent methods to determine bacterial concentration and diversity in freshly caught tropical brackish water shrimps *P. notialis*. Also the relationship between the bacterial concentration and diversity of shrimps and that of their surrounding of water and sediment was investigated. The study contributes to the knowledge of the microbiota of fresh tropical shrimps. It also shows that the overlap between tropical shrimp microbiota and that of their surroundings of brackish water or sediment is low. Thus, bacterial community of tropical brackish water shrimps cannot be predicted from the microbiota of their aquatic environment.

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Supplementary materials

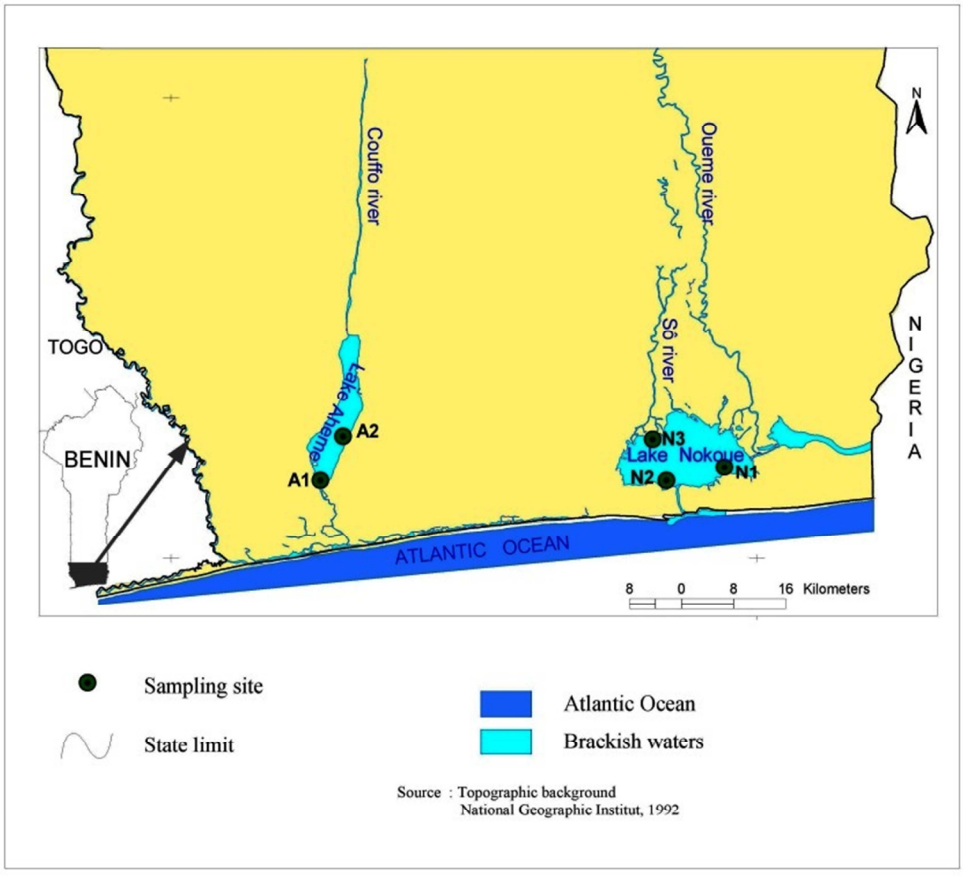

Fig. S3.1 Map of Benin showing the sampling sites.

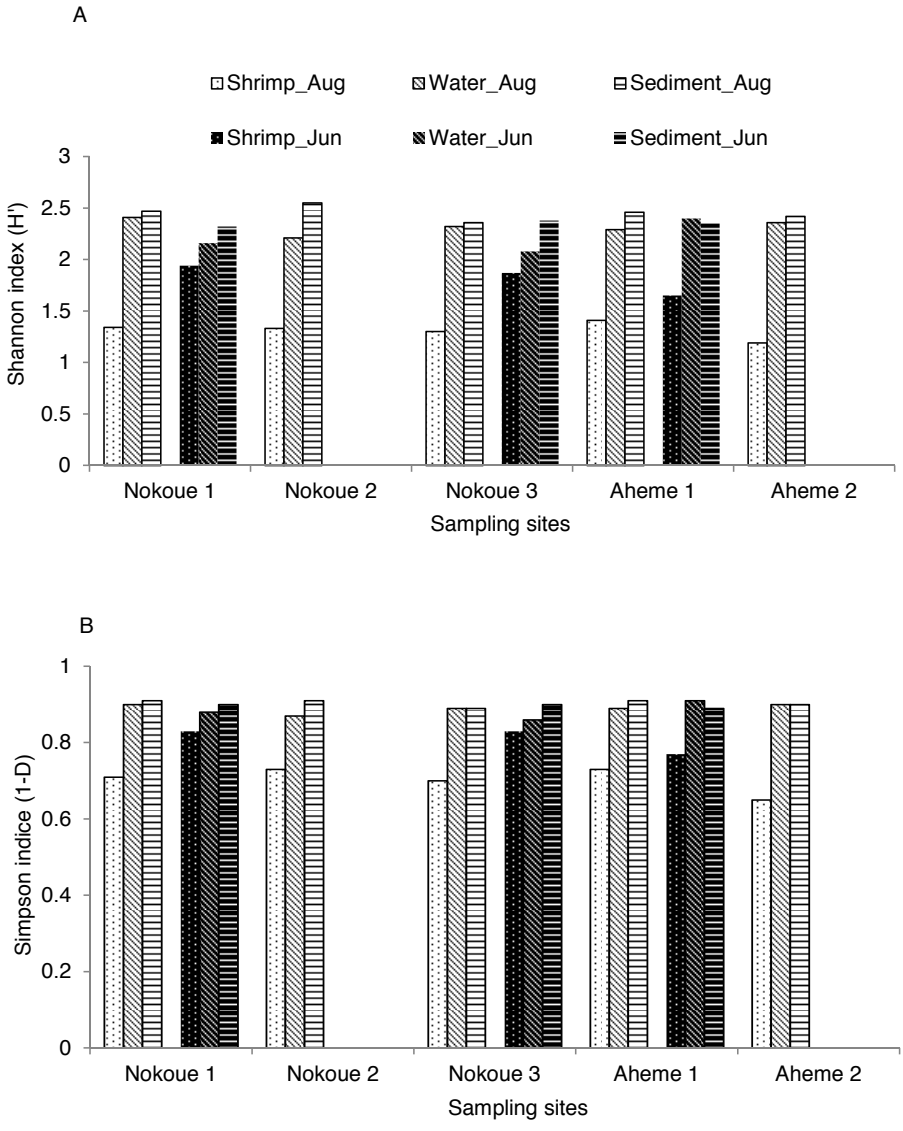

Fig. S3.2 Shannon (A) and Simpson (B) indices of bacterial diversity in shrimp, water and sediment samples based on PCR-DGGE analysis.

[a]**Table S3.1**

Identities of OTUs belonging to the water and the sediment clone libraries

Phylum or class	Clones code	Samples from which clones were recovered[b]	% Similarity and GenBank closest relatives		GenBank accession number
Firmicutes	OTU4	WN1-Jun (23)	99%	*Exiguobacterium indicum*	KP181749
	OTU12	WN1-Jun (6)	99%	*Exiguobacterium aestuarii*	KP181873
	OTU25	WN1-Jun (2)	100%	*Fictibacillus* sp.	KP181921
	OTU35	WN1-Jun (2)	99%	*Planococcus* sp.	KP181939
	OTU41	WN1-Jun (2)	99%	*Bacillus horikoshii*	KP181951
	OTU89	WN1-Jun (1)	99%	*Planococcus maritimus*	KP181997
	OTU91	WN1-Jun (1)	99%	*Bacillus aquimaris*	KP181999
	OTU103	WN1-Jun (1)	99%	*Bacillus baekryungensis*	KP182011
	OTU137	WN1-Jun (1)	99%	*Bacillus* sp.	KP182042
	OTU139	WN1-Jun (1)	99%	uncultured bacterium clone SSB0301-05	KP182044
	OTU159	WN1-Jun (1)	100%	*Bacillus* sp.	KP182059
Alphaprot.	OTU17	WN1-Jun (4)	98%	uncultured bacterium clone HP1B64	KP181897
	OTU30	SeN1-Jun (2)	99%	uncultured bacterium clone STU43	KP181931
	OTU45	SeN1-Jun (1)	98%	*Erythrobacter* sp.	KP181958
	OTU51	SeN1-Jun (1)	98%	uncultured bacterium clone MgKD02d007A07	KP181962
	OTU99	SeN1-Jun (1)	99%	*Mesorhizobium* sp.	KP182007
	OTU105	WN1-Jun (1)	98%	uncultured bacterium clone S15	KP182013
	OTU125	WN1-Jun (1)	98%	uncultured alphaproteobacterium clone 4605-27F	KP182030
	OTU126	WN1-Jun (1)	99%	*Roseobacter* sp.	KP182031
Betaprot.	OTU14	SeN1-Jun (5)	99%	uncultured bacterium clone SZB2	KP181884
	OTU18	SeN1-Jun (3)	97%	uncultured *Alcaligenaceae* AUVE 01E05	KP181901
	OTU58	WN1-Jun (1)	98%	uncultured *Hydrogenophaga* sp.	KP181969
	OTU106	WN1-Jun (1)	99%	uncultured bacterium clone 16S M3 15	KP182014
	OTU124	SeN1-Jun (1)	98%	uncultured bacterium clone D23	KP182029
	OTU158	WN1-Jun (1)	99%	uncultured bacterium clone E70	KP182058
Deltaprot.	OTU56	SeN1-Jun (1)	99%	uncultured *Desulfuromonadales* clone ZL44	KP181967
	OTU108	SeN1-Jun (1)	99%	uncultured deltaproteobacterium clone KorMud-V8C77	KP182016
	OTU111	SeN1-Jun (1)	98%	uncultured bacterium clone NSED2 77	KP182018
	OTU138	SeN1-Jun (1)	99%	uncultured deltaproteobacterium clone RII-AN079	KP182043
	OTU145	SeN1-Jun (1)	97%	uncultured bacterium clone LL3a G05	KP182049
	OTU153	SeN1-Jun (1)	97%	uncultured deltaproteobacterium clone Lake Centre12	KP182055
Gammaprot.	**OTU19**	**WN1-Jun (1) SeN1-Jun (2)**	**98%**	**uncultured *Xanthomonadales* Plot29-2F11**	KP181904
	OTU23	SeN1-Jun (3)	97%	uncultured *Chromatiales* TDNP Wbc97 11 7 16	KP181916
	OTU28	SeN1-Jun (2)	99%	uncultured bacterium clone SSOTU62	KP181927
	OTU43	SeN1-Jun (2)	97%	uncultured bacterium clone Er-MS-109	KP181955
	OTU54	WN1-Jun (1)	99%	*Psychrobacter psychrophilus*	KP181965
	OTU80	SeN1-Jun (1)	99%	uncultured bacterium clone BJGMM-1s-139	KP181988
	OTU101	WN1-Jun (1)	99%	*Pseudomonas plecoglossicida*	KP182009
	OTU118	SeN1-Jun (1)	98%	uncultured *Chromatiales* TDNP Wbc97 24 4 116	KP182024
	OTU134	SeN1-Jun (1)	99%	uncultured organism clone SBYZ 2998	KP182039
	OTU135	SeN1-Jun (1)	99%	uncultured bacterium clone SP-A1-44	KP182040
	OTU144	SeN1-Jun (1)	97%	*Halochromatium* sp.	KP182048

[a]**Table S3.1**

Identities of OTUs belonging to the water and the sediment clone libraries (*continued*)

Phylum or class	Clones code	Samples from which clones were recovered[b]	% Similarity and GenBank closest relatives		GenBank accession number
Acidobacteria	OTU37	SeN1-Jun (2)	99%	uncultured bacterium clone 101-060725 Rokkaku 1	KP181943
	OTU52	SeN1-Jun (1)	99%	uncultured *Acidobacteria* bacterium clone XME61	KP181963
	OTU57	SeN1-Jun (1)	97%	uncultured bacterium clone MidBa14	KP181968
	OTU141	SeN1-Jun (1)	98%	uncultured *Acidobacterium* sp. clone MS-N36	KP182046
Actinobact.	OTU29	WN1-Jun (2)	99%	uncultured marine bacterium clone S24-66	KP181929
	OTU36	WN1-Jun (2)	99%	uncultured bacterium clone 5C231574	KP181941
	OTU40	WN1-Jun (2)	99%	uncultured *Actinomycetales* SHWH night1 16S489	KP181949
	OTU55	WN1-Jun (1)	97%	*Mycobacterium fallax*	KP181966
	OTU70	WN1-Jun (1)	100%	*Rhodococcus qingshengii*	KP181979
	OTU77	WN1-Jun (1)	99%	uncultured bacterium clone dcfb4-68	KP181985
	OTU82	WN1-Jun (1)	100%	uncultured bacterium clone Contig\97	KP181990
	OTU94	WN1-Jun (1)	98%	uncultured bacterium clone caohai-32	KP182002
	OTU114	WN1-Jun (1)	99%	uncultured actinobacterium clone CB01F02	KP182021
	OTU128	WN1-Jun (1)	98%	Candidatus *Rhodoluna lacicola*	KP182033
Chloroflexi	OTU38	SeN1-Jun (2)	99%	uncultured Chloroflexi clone QEDQ1CA04	KP181945
	OTU84	SeN1-Jun (1)	97%	uncultured *Caldilinea* sp. clone CFX166	KP181992
	OTU92	SeN1-Jun (1)	97%	uncultured *Anaerolineaceae* SRO176E01c	KP182000
	OTU123	SeN1-Jun (1)	99%	uncultured bacterium clone HKB1	KP182028
Cyanobact.	OTU13	WN1-Jun (5)	99%	*Cyanobium* sp.	KP181879
	OTU65	WN1-Jun (1)	98%	uncultured *Synechococcus* sp. clone BAN2-04	KP181975
	OTU133	SeN1-Jun (1)	99%	*Pennate diatom* sp. CCAP 1008/1	KP182038
Deinococ.-T	OTU39	SeN1-Jun (2)	98%	uncultured *Thermales* clone A1943	KP181947
Gemmatimo.	OTU149	SeN1-Jun (1)	97%	uncultured *Gemmatimonadetes* clone VERDEA61	KP182053
Planctomyc.	OTU46	WN1-Jun (1)	99%	uncultured bacterium clone E06LBA	KP181959
	OTU60	SeN1-Jun (1)	97%	uncultured *Planctomyces* clone 0907 Mf DT2	KP181970
	OTU79	WN1-Jun (1)	99%	uncultured *Planctomycetes* clone QEEA3DA09	KP181987
	OTU112	SeN1-Jun (1)	99%	uncultured *Planctomycetes* clone IPI 1541-1637-1733	KP182019
	OTU136	WN1-Jun (1)	99%	uncultured bacterium clone 0.1m-1097-27	KP182041
Verrucomic.	OTU93	WN1-Jun (1)	99%	uncultured prokaryote clone Ser1-21	KP182001
	OTU148	WN1-Jun (1)	99%	uncultured *Verrucomicrobiaceae* clone Gap-4-68	KP182052
	OTU150	WN1-Jun (1)	99%	uncultured bacterium clone S0054	KP182054

[a]The OTU shared between the water and sediment is shown in bold.

[b]Numbers in parenthesis indicate the number of clones.

Se = sediment, W = water, Sh = shrimp, Ni= site i in Lake Nokoue, Ai = site i in Lake Aheme, Jun = sampling period (June)

Alphaprot. = *Alphaproteobacteria*, *Betaprot.* = *Betaproteobacteria*, *Deltaprot.* = *Deltaproteobacteria*, *Gammaprot.* = *Gammaproteobacteria*, *Actinobact.* = *Actinobacteria*, *Cyanobact.* = *Cyanobacteria/Chloroplast*, *Deinococ.-T* = *Deinococcus-Thermus*, *Gemmatimo.* = *Gemmatimonadetes*, *Planctomyc.* = *Planctomycetes*, *Verrucomic.* = *Verrucomicrobia*.

4

Spoilage evaluation, shelf-life prediction, and potential spoilage organisms of tropical brackish water shrimp (*Penaeus notialis*) at different storage temperatures

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Abstract

*Maintaining the freshness of shrimp is a concern to shrimp stakeholders. To improve shrimp quality management, it is of importance to evaluate shrimp spoilage characteristics. Therefore, microbiological, sensory, and chemical changes of naturally contaminated tropical brackish water shrimp (*Penaeus notialis*) during storage at 28ºC, 7ºC and 0ºC were assessed. H_2S-producing bacteria were the dominant group of microorganisms at 28ºC and 7ºC whereas* Pseudomonas *spp. were dominant at 0ºC. Total volatile basic nitrogen and trimethylamine correlated well (R^2 >0.90) with the sensory scores. An empirical model to predict the shelf-life of naturally contaminated tropical shrimp as a function of storage temperature was developed. Specific groups of organisms were isolated at the sensory rejection times and assessed for spoilage potential in shrimps of which the endogenous flora was heat inactivated. Isolates capable of producing strong off-odor identified by 16S rRNA sequencing were mainly lactic acid bacteria (LAB) and* Enterobacteriaceae *at 28ºC or 7ºC and* Pseudomonas *spp. and LAB at 0ºC. The study contributes to the knowledge about tropical shrimp spoilage and provides a basis for the development of methods and tools to improve shrimp quality management.*

Introduction

Shrimp is one of the most traded and consumed aquatic products worldwide (Chen et al., 2013; Wan Norhana et al., 2010). In Benin (West Africa), shrimps are caught from brackish waters (lakes and lagoons), processed and sold in the local market or exported (Dabadé et al., 2014). Shrimp is prone to deterioration because of its high content of free amino-acids and other soluble non-nitrogenous substances, which can serve as easily digestible nutrients for microbial growth (Zeng et al., 2005). However, only a few members of the microbial community of freshly caught or processed seafood, the so-called specific spoilage organisms (SSOs) really contribute to the seafood spoilage (Gram and Dalgaard, 2002). The identification of bacteria that are responsible for spoilage requires sensory, microbiological and chemical studies (Gram and Huss, 1996). Specific spoilage organisms produce various volatile compounds, some of which could function as indicators of spoilage. Volatile amines, including trimethylamine (TMA), ammonia (NH_3) and dimethylamine (DMA) represent total volatile basic nitrogen (TVB-N), which is the most widely used parameter to reveal microbiological spoilage of seafood (Chan et al., 2006; Pacquit et al., 2007).

Heinsz et al. (1988) reported that *Acinetobacter* spp. were the main organisms responsible for the spoilage of shrimps (*Penaeus aztecus*) harvested from Georgia coastal waters. The dominant microorganisms in shrimps (*Penaeus merguiensis*) harvested from Pakistan at sensory rejection times were found to be *Moraxella* spp. at low storage temperatures (0-10ºC) and *Vibrio* spp. at high storage temperatures (15-35ºC) (Shamshad et al., 1990). Assessing the spoilage pattern of five species of shrimps, Chinivasagam et al. (1996) found that *Pseudomonas fragi* was the main spoiler of iced-stored tropical shrimps, and *Shewanella putrefaciens*, the dominant microorganism in tropical shrimps stored in ice slurry. All these reports on shrimp spoilage showed that even for the same seafood product, spoilage may develop differently, depending on geographical origin and other unknown factors interacting with microbial growth (Gram and Huss, 1996).

To our knowledge, there is no report on specific spoilage organisms of tropical shrimp caught from brackish waters in Africa. The aim of this work was to (i) evaluate the quality changes of shrimps caught from brackish waters stored at different temperatures and the effect of storage temperature on their shelf-life, (ii) assess the spoilage potential of bacterial strains isolated at sensory rejection time, and (iii) identify the major spoilage bacteria.

Materials and methods

Samples preparation and storage conditions

Freshly caught shrimps (*Penaeus notialis*) were collected from Lake Nokoue and Lake Aheme in Benin (West Africa), which are the most important shrimp fishing areas in Benin. The average weights of shrimps from Lake Nokoue and Lake Aheme were 12±2 g and 20±3 g per shrimp, respectively. Immediately after collection, samples were cooled with ice and transferred to the laboratory within 2 h. Packs of shrimps were put in polyethylene bags and stored at 0ºC, 7ºC and 28ºC. For 0ºC storage, the method by Shamshad et al. (1990) was used. Shrimps were put in ice in a plastic container with drain holes. The plastic container was put on another container and kept at 4ºC. The ratio of shrimp to ice was approximately 1:3. Shrimps were re-iced daily to maintain the same ratio. For 7ºC and 28ºC storage, shrimps were stored in Sanyo MIR-153 refrigerated and Memmert incubators, respectively. For all storage temperatures, thermochron iButton (DS 1921G) devices were placed in some shrimp samples to record temperatures during storage. At appropriate time intervals, three packs of shrimp were taken out and each pack was used for microbiological, chemical and sensory analyses to obtain triplicate analysis per sampling time point. All microbiological media and chemicals used were from OXOID and SIGMA, respectively.

Microbiological analysis

Whole shrimps (2-3 individuals per pack) were aseptically cut into small pieces, after which a 25 g sample was transferred aseptically to a stomacher bag and diluted 10 times in physiological saline peptone solution (0.85% NaCl, 0.1% peptone). The mixture was homogenized for 60 s using a stomacher (Seward Laboratory Stomacher 400, England) to get the first dilution from which successive decimal dilutions were prepared. Total Viable Counts (TVC) measured as aerobic plate counts, and H_2S-producing bacteria were enumerated on double layered plates of iron agar (IA) supplemented with 0.04% L-cysteine and incubated at 25ºC for 72 h as described by Gram et al. (1987). Black colonies were counted as H_2S-producing bacteria and TVC as the sum of black and white colonies as previously described (Broekaert et al., 2011; Giuffrida et al., 2013; Mai et al., 2011; Martinez-Alvarez et al., 2009). *Enterobacteriaceae* were enumerated on double-layered plates of violet red bile glucose (VRBG) medium and incubated at 37ºC for 24 h. *Pseudomonas* spp. were enumerated on spread plates of *Pseudomonas* agar base supplemented with cetrimide, fucidin, and cephaloridine (CFC) at 25ºC for 48 h. Lactic acid bacteria (LAB) were enumerated on double-layered plates of de Man, Rogosa and Sharp agar (MRSA) incubated at 30ºC for 72 h. For the double-layered plates, 1 mL of the appropriate dilution was inoculated into a Petri dish, then approximately 15 mL of the molten (45°C) medium was poured into the Petri dish. After setting, the Petri dish was overlaid with approximately 10 mL of the same molten medium.

Chemical analysis

Total volatile basic nitrogen (TVBN), trimethylamine (TMA) and pH were determined. TVBN (mg/100 g shrimp) was measured using the method recommended by the European Commission (EC, 2005). Briefly, 10 g of ground shrimp were homogenized with 90 mL 6% perchloric acid (SIGMA) solution for 2 min. After filtering, 50 mL of the extract were alkalinized with 20% sodium hydroxide, and submitted to steam distillation. The distillation apparatus was set to produce approximately 100 mL of distillate in 10 min. The volatile base components absorbed by boric acid solution (3%) in a beaker were determined by titration using hydrochloric acid solution (0.01 mol). To determine TMA, 20 mL of 35% formaldehyde (an alkaline binding mono and diamine) was added to 25 mL perchloric acid shrimp extract prior to the following steps. Thus, TMA was the only volatile and measurable amine (Magnusson et al., 2006; Malle and Tao, 1987). To determine the pH, 20 mL of distilled water were added to 10 g of ground shrimp. The mixture was homogenized and the pH was measured in duplicate using a pH meter (InoLab 730).

Sensory analysis

Ten panelists experienced in shrimp freshness evaluation carried out the sensory analysis. At each sensory session, the panelist evaluated one piece of whole shrimp from each pack of shrimps. Samples of freshly thawed shrimps were also included to reduce the risk of panelists guessing the development in sensory score (Mejlholm et al., 2005). The odor of raw shrimps was evaluated using a scoring scale with three categories (Argyri et al., 2010; Dalgaard et al., 1993; Mejlholm et al., 2008) corresponding to 1 = fresh (shrimp without any off-odor), 2 = marginal (shrimp having slight off-odor but still being acceptable) and 3 = spoiled (shrimp producing strong off-odor). Time of sensory rejection was defined as the time when at least 50% of the panelists evaluated samples to be in category 3.

Effect of storage temperature on the shelf-life of naturally contaminated shrimp

Data on the rates of spoilage determined as the reciprocal of shelf-life (RS, day^{-1}) from both lakes were combined and three empirical models: exponential model (Eq. (4.1)), Ratkowsky model (Eq. (4.2)) (Ratkowsky et al., 1982) and Arrhenius model (Eq. (4.3)) were fitted to the combined RS-data.

$$RS = b_1 \times \exp(a \times T) \tag{4.1}$$

$$RS = {b_2}^2 \times (T - T_{\min})^2 \tag{4.2}$$

$$RS = b_3 \times \exp\left(\frac{-E_a}{R \times K}\right) \tag{4.3}$$

with T is the storage temperature (ºC), R is the gas constant 8.314 (J mol^{-1} K^{-1}), K the temperature in Kelvin. b_i, a, T_{min}, and E_a are the coefficients to be estimated.

The variance of *RS* was high at high storage temperatures. Therefore, RS-data were ln-transformed to stabilize the variance over the temperature range. The performance of the three models was assessed by fitting the models to the ln-transformed data and comparing the root mean square error of the model ($RMSE_{model}$) as described by Den Besten et al. (2006).

$$RMSE_{\text{model}} = \sqrt{\frac{\sum (\text{observed}_i - \text{fitted}_i)^2}{n - s}} \tag{4.4}$$

observed_i, are the observed values, fitted_i, are the described values, n is the number of data points, s is the number of parameters of the model.

Spoilage potential assessment

At sensory rejection times, two counted plates (20-200 colonies per plate) were randomly selected per group of microorganisms at each storage temperature. At least 5 colonies per plate, representative of the variability in shape, color and size of all colonies in the plate, were selected. The colonies were checked for purity on MRSA for lactic acid bacteria and on tryptone soya agar (TSA) for other groups of microorganisms. Presumptive tests namely Gram staining and catalase test, oxidase and glucose fermentation test, and oxidase test were performed on lactic acid bacteria, *Enterobacteriaceae*, and *Pseudomonas* spp., respectively as previously described (Rodriguez-Calleja et al., 2012; Zheng et al., 2012). Pure cultures of isolated colonies were inoculated to freshly caught shrimp of which the endogenous flora was heat inactivated at 100ºC for 30 min. The inoculation was approximately 10^5 CFU/g of shrimp. The samples were stored as described above at the temperature of initial isolation. The ability of the inoculated culture to produce off-odor was evaluated by the same panelists. Strains deemed to produce strong off-odor by at least 50% of the panelists were identified following the procedure described below.

Identification of spoilage bacteria

Isolates capable of producing strong off-odor were grown on TSA plates overnight. The protocol described in the genomic DNA purification kit (Promega Corporation) was used to extract DNA. The DNA extracts were used to amplify the 16S rRNA gene with polymerase chain reaction (PCR) using the thermocycler GeneAmp PCR system 9700 (Applied Biosystems). The PCR was performed using 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') universal primers with PCR conditions as follows: initial denaturation of double stranded DNA at 94ºC for 5 min, then 35 amplification cycles with denaturation at 94ºC for 20 s, primer annealing at 56ºC for 20 s, and extension at 72ºC for 1 min, finally an extension at 72ºC for 7 min followed by cooling at 4ºC. The PCR products were sequenced at GATC Biotech (Germany). The DNA Baser Sequence Assembler v3.5.2 (2012) (http://www.DnaBaser.com) was used to assemble the forward and reverse sequences. The sequences were checked with Bellerophon's chimera detection program (Hubert et al., 2004) and compared against the GenBank database using the basic local alignment search tool (BLAST) (Altschul et al., 1990). The sequences were deposited in the GenBank database under the accession numbers KJ626227-KJ626264.

Results

The actual storage temperatures recorded were 27.8±0.4ºC, 7.1±0.3ºC, and 0.1±0.3ºC during storage at 28ºC, 7ºC, and 0ºC respectively. The results obtained in the present work with shrimps from Lake Nokoue and Lake Aheme were rather similar. Therefore, we present here the results of shrimps from Lake Aheme and data from Lake Nokoue are included as supplemental data. However, the identified spoilage organisms and data used for shelf-life prediction were from shrimps caught in both lakes.

Microbiological analysis

The evolution of TVC, H_2S-producing bacteria, *Pseudomonas* spp., *Enterobacteriaceae*, and LAB concentration during shrimp storage at different temperatures is shown in Fig. 4.1. The initial concentrations of the different groups of microorganisms varied between 2.7 log CFU/g (*Enterobacteriaceae*) and 5.5 log CFU/g (TVC).

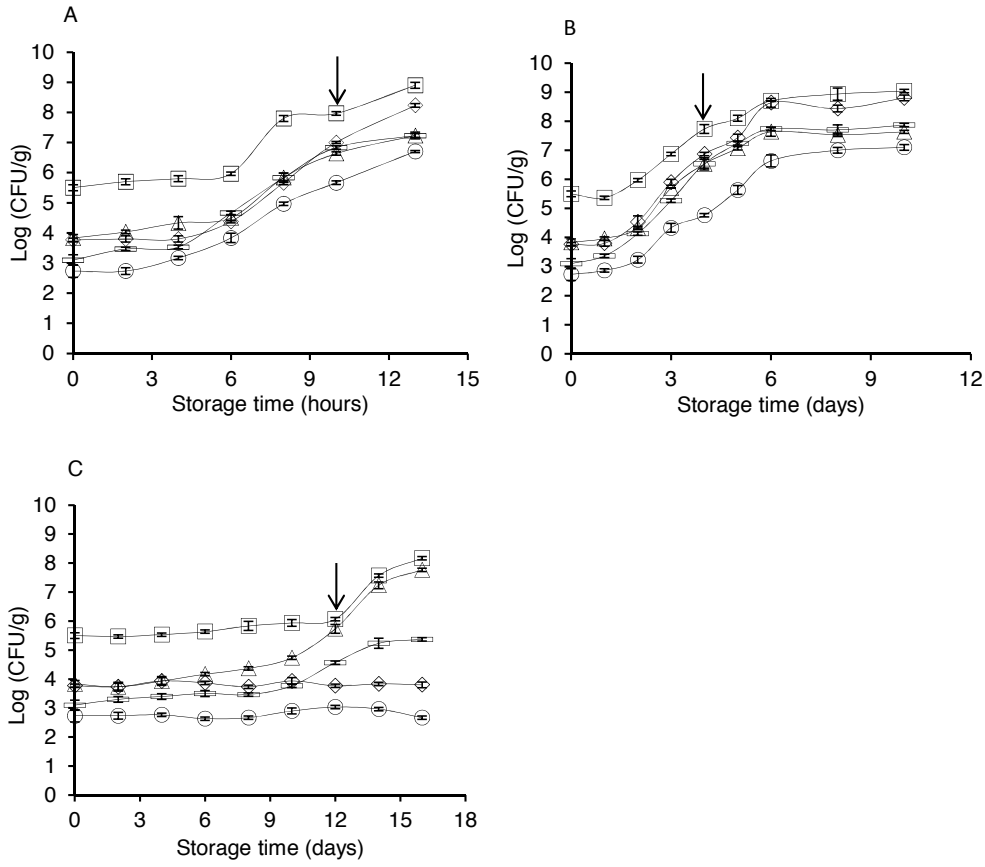

Fig. 4.1 Evolution of total viable counts (□), H_2S-producing bacteria (◇), *Pseudomonas* spp. (△), Enterobacteriaceae (○), and lactic acid bacteria (▭) of shrimp stored at 28ºC (A), 7ºC (B), and 0 C (C). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

During storage at 28ºC, TVC increased slowly the first 6 h, and then rapidly increased to 8.9 log CFU/g after 13 h of storage. H_2S-producing bacteria were the dominant group of microorganisms at the end of storage with a concentration of 8.2 log CFU/g. *Pseudomonas* spp., *Enterobacteriaceae*, and LAB also grew throughout the storage time reaching 7.2, 6.7, and 7.2 log CFU/g, respectively at the end of storage.The growth pattern of the enumerated bacteria in the samples stored at 7ºC was similar but slower than the one observed at 28ºC. After the first day of storage, total viable counts slowly increased to 9.0 log CFU/g by the end of storage. H_2S-producing bacteria were also the dominant group of microorganisms at the end of storage at 7ºC reaching 8.8 log CFU/g. *Pseudomonas* spp. counts increased to their maximal concentration of 7.6 log CFU/g after 6 days of storage and remained constant until the end of storage. *Enterobacteriaceae* and LAB concentrations increased to 7.1 and 7.9 log CFU/g respectively at the end of storage time. In samples stored at 0ºC, TVC proliferated slowly the first 10 days of storage. Afterward, they increased to 8.2 log CFU/g at the end of storage time. *Pseudomonas* spp. were the dominant group of microorganism during storage at 0ºC. At the end of storage, *Pseudomonas* spp. counts increased to 7.4 log CFU/g. LAB counts increased to the level of 5.4 log CFU/g at the end of storage time. Throughout the storage time, H_2S-producing bacteria and *Enterobacteriaceae* counts remained almost constant.

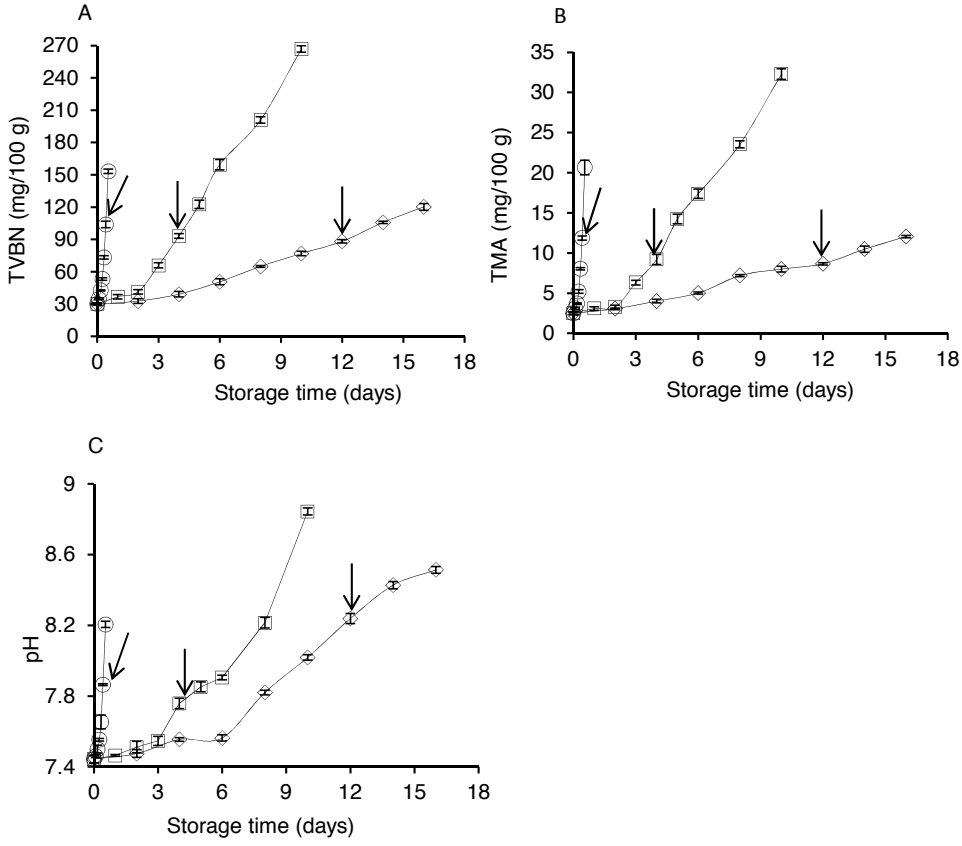

Fig. 4.2 Evolution of total volatile basic nitrogen (TVBN) (A), trimethylamine (TMA) (B), pH (C) of shrimp at 28ºC (○), 7ºC (□), and 0ºC (◇). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

Chemical changes

The evolution of TVBN, TMA and pH values in shrimp samples stored at 28ºC, 7ºC, and 0ºC is depicted in Fig. 4.2A, Fig. 4.2B, and Fig. 4.2C, respectively. The initial values of TVBN, TMA and pH were 30.1 mg/100 g, 2.5 mg/100 g, and 7.4 respectively. TVBN, TMA and pH values increased throughout the storage. As expected, the rate of the chemical indicator change was higher at high temperature than at low temperature. At the sensory rejection times, TVBN values ranged from 88 to104 mg/100 g and TMA values, from 9 to 12 mg/100 g.

Sensory analysis

The results of shrimp sensory evaluation are presented in Table 4.1. At the beginning of storage time, shrimp samples did not produce any off-odor according to 96.7% (mean percentage of three packs of shrimps, each assessed by 10 panelists) of the panelists. The percentage of the panelists evaluating the shrimp samples as producing strong off-odor increased with storage time. The sensory rejection time defined as the time when at least 50% of the panelists evaluate shrimp to produce strong off-odor was 10 h at 28ºC, 4 d at 7ºC, and 12 d at 0ºC.

Table 4.1

Shrimp off-odor evaluation (in % of panelists) during storage at 28ºC (A), 7ºC (B), and 0ºC (C)

A

Odor evaluation	Storage time (hours)						
	0	2	4	6	8	10	13
No off-odor	96.7	93.3	83.3	66.7	10.0	0	0
Slight off-odor	3.3	6.7	16.7	33.0	83.3	46.7	6.7
Strong off-odor	0	0	0	0	6.7	**53.3**	93.3

B

Odor evaluation	Storage time (days)								
	0	1	2	3	4	5	6	8	10
No off-odor	96.7	86.7	80.0	10.0	0	0	0	0	0
Slight off-odor	3.3	13.3	20.0	66.7	43.0	30.0	6.7	0	0
Strong off-odor	0	0	0	23.3	**56.7**	70.0	93.3	100	100

C

Odor evaluation	Storage time (days)								
	0	2	4	6	8	10	12	14	16
No off-odor	96.7	93.3	90.0	30.0	10.0	6.7	6.7	0	0
Slight off-odor	3.3	6.7	10.0	70.0	86.7	83.3	43.0	40.0	33.3
Strong off-odor	0	0	0	0	3.3	10.0	**50.0**	60.0	66.7

Values in boldface indicate sensory rejection times.

Relationship between chemical indicators and microbial concentration or sensory scores

At all storage temperatures, from the beginning of storage until the sensory rejection time, a good correlation (R^2 >0.90) was found between TVBN or TMA production and the means of sensory scores. (Fig. 4.3A and Fig. 4.3B). The correlation between pH values and the sensory scores was lower (Fig. 4.3C). A good but lower correlation (R^2 >0.85) was found between TVBN production and the log counts of TVC or the dominant group of microorganisms at the different storage temperatures (data not shown).

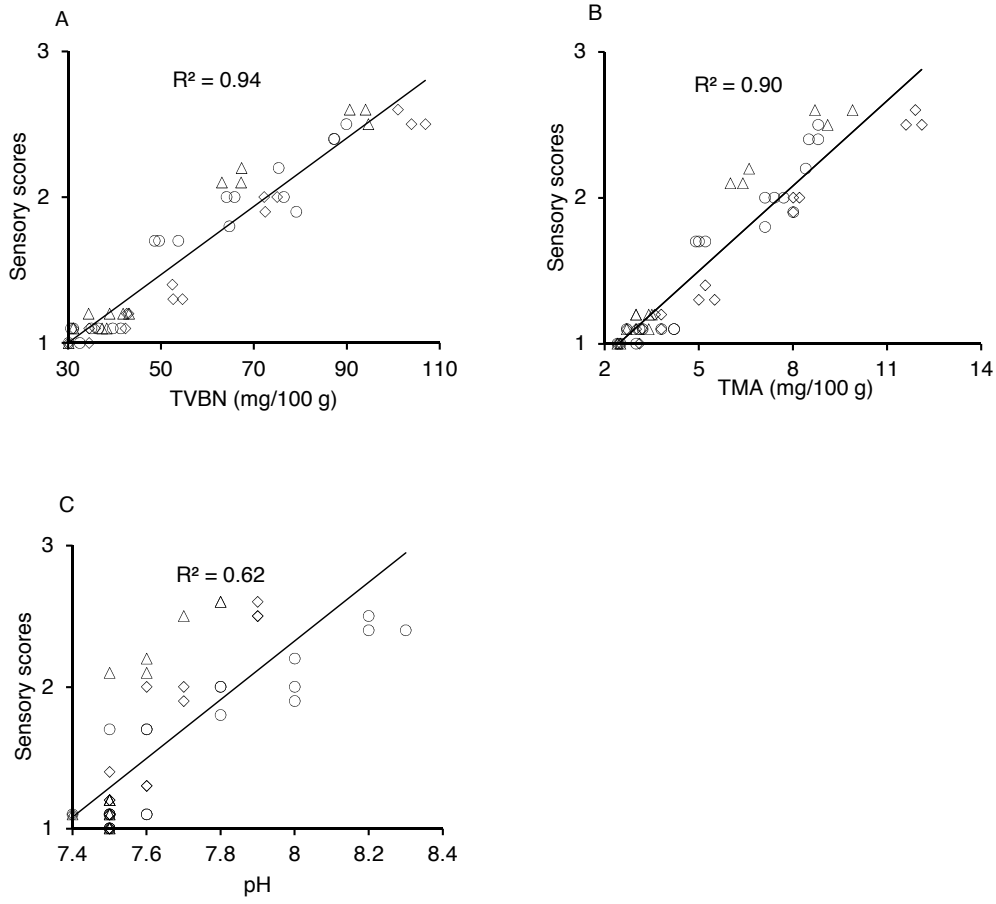

Fig. 4.3 Relationship between sensory scores and total volatile basic nitrogen (TVBN) (A), trimethylamine (TMA) (B), and pH (C) values from the beginning of shrimp storage at 28ºC (◇), 7ºC (△) and 0ºC (○) until the sensory rejection time.

Sensory scores: 1= shrimp without any off-odor, 2 = shrimp having slight off-odor but still being acceptable and 3 = shrimp producing strong off-odor. The average of 10 panelists was used.

Effect of temperature on spoilage rate and shelf-life prediction

The root mean square error of ($RMSE_{model}$) of exponential, Ratkowsky, and Arrhenius models were 0.18, 0.21, and 0.15, respectively. The best fitting model was the Arrhenius model and the fitted parameters E_a and $\ln b_3$ were 80.2±4.5 kJ mol^{-1} (95% CI) and 33.03±1.89 (95% CI), respectively. The effect of temperature on the rate of spoilage is shown in Fig. 4.4.

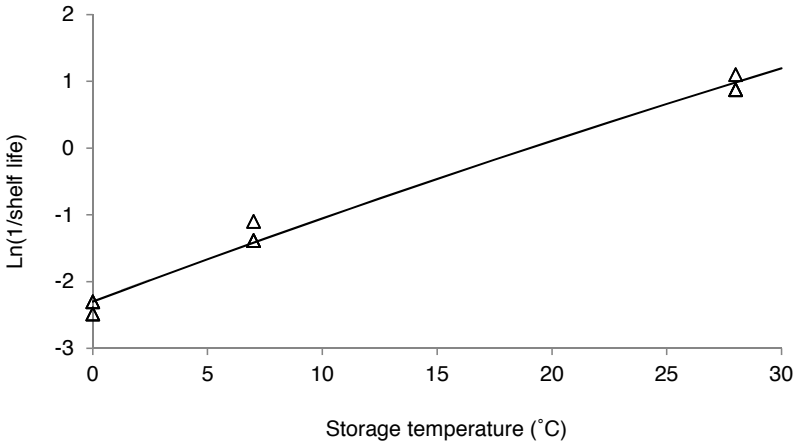

Fig. 4.4 Shrimp shelf-life modeled with Arrhenius model using data from Lake Nokoue and Lake Aheme
Legend: time is in days.

With the estimated parameters E_a and b_3 (2.21×10^{14}) the shelf-life of tropical brackish water shrimp *P. notialis* could be predicted at a given storage temperature using Eq. (4.5)

$$\text{Shelf-life (days)} = 4.5\times10^{-15}\times\exp\left(\frac{9650}{K}\right) \quad (4.5)$$

with K, the storage temperature in Kelvin.

Spoilage potential assessment and identification of spoilage bacteria

A total of 38 out of 189 strains isolated at sensory rejection time and assessed for spoilage potential (their ability to produce off-odor) were detected to produce strong off-odor. The spoilage assessment results and the identity of isolates capable of producing strong off-odor are shown in Table 4.2. The strongest off-odor producers belonged to the *Enterobacteriaceae* and lactic acid bacteria at 28ºC and 7ºC, whereas at 0ºC the off-odor producing bacteria were *Pseudomonas* spp. and lactic acid bacteria. It should be noted that all the black colonies isolated from IA were identified as members of lactic acid bacteria. *Morganella morganii*, a member of *Enterobacteriaceae*, was twice isolated from *Pseudomonas* agar in this study.

Discussion

The present study aimed at evaluating the quality change of tropical brackish water shrimps (*P. notialis*). The initial total aerobic counts obtained in this study (5.5 log CFU/g) are higher than those reported by Chinivasagam et al. (1996), Heinsz et al. (1988), Mendes et al. (2002) in cold-water shrimp, but close to 5.7 log CFU/g reported by Shamshad et al. (1990) in tropical shrimp. This confirms that in general, tropical shrimps carry higher initial numbers of bacteria, 10^5-10^6 CFU/g, than cold-water species, 10^2-10^4 CFU/g (ICMSF, 2005).

Hydrogen sulfide-producing bacteria, which were found in this study to be the dominant group of microorganisms during storage at 7ºC and 28ºC, are well known as seafood spoilage bacteria. H_2S-producing bacteria constitute the most relevant specific spoilage organisms in aerobically stored and vacuum packed marine fish (Skjerdal et al., 2004). One way to quantify H_2S-producing bacteria in seafood is the use of IA supplemented with cysteine (Gram et al., 1987). Bacteria able to produce hydrogen sulfide (H_2S) when decomposing thiosulfate or cysteine present in IA medium, form black colonies due to precipitation of iron

sulfide (FeS) (Gram et al., 1987). According to Skjerdal et al. (2004), this way of detecting H_2S-producing bacteria has an advantage of reducing the risk of detecting false positives and negatives since the detection reaction is directly related to the spoilage property of H_2S-producing bacteria. It has been reported that H_2S-producing bacteria in seafood products are composed mainly of *Shewanella baltica* (Serio et al., 2014; Vogel et al., 2005), *Shewanella morhuae* (Serio et al., 2014), and *Shewanella putrefaciens* (Chinivasagam et al., 1996; Gram et al., 1987; Koutsoumanis and Nychas, 1999; Mohan et al., 2010). The latter has been identified as the specific spoilage organism of iced marine fish (Gram and Dalgaard, 2002). However, in the present work, black colonies capable of producing strong off-odor isolated from iron agar and purified on TSA were identified by 16S rRNA sequencing as lactic acid bacteria (LAB). Our finding suggests that LAB are an important member of H_2S-producing spoilage bacteria associated with tropical warm-water shrimp storage. The ability of some LAB to produce H_2S in food has been documented. For example, *Lactobacillus sake* spoils vacuum-packaged beef (Egan et al., 1989) and cold-smoked salmon (Joffraud et al., 2001) by producing H_2S. *Vagococcus* species have been isolated from the microbiota of spoiled tropical cooked shrimp (*Penaeus vannamei*) (Jaffre et al., 2009). The authors of this work pointed out that it was the first time that the genus *Vagococcus* was isolated from a spoilage microbiota. However, they did not assess the spoilage potential of the isolates. In fact, not all members of spoilage microbiota of a product necessarily contribute to the spoilage of that product (Gram and Dalgaard, 2002). The present work showed that *Vagococcus fluvialis* is not only a member of spoilage microbiota of tropical shrimp but also a potential spoilage organism since it can produce H_2S and strong off-odor. *Lactococcus garvieae* was also isolated from spoilage microbiota of warm-water cooked and brined shrimp (Dalgaard et al., 2003).

Other spoilage LAB isolated from the same iron agar medium but as white colonies were *Enterococcus faecalis* (7ºC and 0ºC) and *Carnobacterium maltaromaticum*. *Carnobacterium* species have been reported to be the dominant part of spoilage association of cooked and brined modified atmosphere packed shrimps stored at 0ºC (Dalgaard et al., 2003). Mace et al. (2014) found that *C. maltaromaticum* is responsible for strong and rapid spoilage of cooked whole tropical shrimp stored under modified atmosphere packaging. *E. faecalis* has been isolated from the spoilage microbiota of shrimp stored at or above 15ºC (Dalgaard et al., 2003; Jaffres et al., 2009) and in the present study, the isolated *E. faecalis* was able to produce strong off-odor at 7ºC and 0ºC.

Apart from one isolate identified as *Weissella cibaria*, all the spoilage LAB in the present work were not isolated from MRSA but from IA. This could be justified by the fact that some LAB, mainly *Carnobacterium* species, grow poorly in MRSA due to the acetate contained in this medium (Leroi, 2010). In addition, the pH of MRSA (6.2) which is lower than the pH of IA (7.4) could be another reason. In fact, Peirson et al. (2003) found that LAB that cause green discoloration in cooked cured meat grew well in mildly alkaline broth but failed to grow at pH 5.4. They concluded that greening in cooked cured meat may be due to alkalitrophic LAB and not to acidophilic LAB. Finally, the presence of cysteine in IA may have stimulated the growth of spoilage LAB since it is known that some LAB require cysteine for growth (Seefeldt and Weimer, 2000).

Our results on microbiological changes in shrimp during storage at 0ºC are in agreement with earlier observations that *Pseudomonas* spp. are the dominant microorganisms in tropical shrimps stored in ice (Chinivasagam et al., 1996; Chinivasagam et al., 1998; Jeyasekaran et al., 2006). According to Yumoto et al. (2001), *Pseudomonas psychrophila* was previously known as *Pseudomonas* sp. strain E-3. These authors found that strain E-3 was closest to *Pseudomonas fragi* based on 16S rRNA sequencing but low level of homology was found between them based on DNA-DNA hybridization. Therefore, they concluded that strain E-3 was an individual species and proposed the name *Pseudomonas psychrophila*.

High storage temperature (28ºC) was considered in this study to reflect the situation of ambient temperature storage of shrimp, which sometimes occurs in Benin. Our results showed that high storage temperatures (7ºC and mainly 28ºC) allow *Enterobacteriaceae* to produce strong off-odor when inoculated as pure culture in sterile shrimp. *Enterobacteriaceae* are known to produce indole and putrescine, which are

considered as indicators of decomposition in shrimp (Bener et al., 2004; Mendes et al., 2002; Mendes et al., 2005; Wunderlichova et al., 2013).

Table 4.2

Spoilage potential assessment and identities of isolates capable of producing strong off-odor at 28ºC, 7ºC and 0ºC

T[1] (ºC)	Culture media	Number of isolates tested	Number of isolates producing strong off-odor	% Similarity and GeneBank closest relatives	GenBank given accession number	% of panelists judging the isolates to produce strong off-odor (n = 10)
28	Iron Agar (black colonies)	15	5	99% *Lactococcus garvieae*	KJ626245	80
				99% *Lactococcus garvieae*	KJ626247	90
				99% *Lactococcus garvieae*	KJ626248	80
				99% *Lactococcus garvieae*[*]	KJ626234	80
				97% *Enterococcus aquimarinus*	KJ626244	70
	Iron Agar (white colonies)	16	3	99% *Morganella morganii*	KJ626255	60
				99% *Lactococcus garvieae*	KJ626257	80
				97% *Acinetobacter calcoaceticus*[*]	KJ626230	70
	PSA[2]	14	1	99% *Morganella morganii*	KJ626253	50
	VRBGA[3]	15	11	99% *Klebsiella pneumoniae*	KJ626250	70
				99% *Klebsiella pneumoniae*	KJ626261	80
				99% *Klebsiella pneumonia*[*]	KJ626227	70
				100% *Klebsiella variicola*[*]	KJ626231	80
				99% *Escherichia fergusonii*	KJ626246	80
				99% *Escherichia fergusonii*	KJ626262	90
				99% *Escherichia fergusonii*	KJ626263	80
				99% *Shigella sonnei*[*]	KJ626232	80
				97% *Proteus vulgaris*	KJ626254	80
				99% *Escherichia fergusonii*	KJ626264	70
				99% *Providencia vermicola*[*]	KJ626236	60
	MRSA[4]	12	0	-	-	-
7	Iron Agar (black colonies)	12	5	99% *Vagococcus fluvialis*	KJ626239	60
				99% *Vagococcus fluvialis*	KJ626241	60
				99% *Vagococcus fluvialis*	KJ626242	60
				99% *Vagococcus fluvialis*	KJ626243	70
				99% *Enterococcus faecalis*	KJ626240	70
	Iron Agar (white colonies)	13	2	99% *Proteus mirabilis*	KJ626258	70
				99% *Acinetobacter johnsonii*[*]	KJ626237	60
	PSA	12	1	99% *Morganella morganii*	KJ626252	60
	VRBGA	11	4	99% *Proteus penneri*[*]	KJ626233	80
				99% *Proteus penneri*[*]	KJ626235	70
				99% *Proteus penneri*[*]	KJ626228	70
				99% *Proteus penneri*[*]	KJ626229	80
	MRSA	11	1	99% *Weissella cibaria*	KJ626260	70
0	Iron Agar (black colonies)	12	0	-	-	-
	Iron Agar (white colonies)	13	3	99% *Pseudomonas gessardii*	KJ626259	100
				99% *Enterococcus faecalis*	KJ626256	80
				99% *Carnobacterium maltaromaticum*	KJ626238	100
	PSA	12	2	99% *Pseudomonas psychrophila*	KJ626249	100
				99% *Pseudomonas gessardii*	KJ626251	90
	VRBGA	10	0	-	-	-
	MRSA	11	0	-	-	-
	Total	**189**	**38**			

[1]Temperature, the strains were isolated from naturally contaminated shrimps and assessed for spoilage potential at the same storage temperature; [2]*Pseudomonas* selective agar; [3]Violet red bile glucose agar; [4]de Man, Rogosa and Sharp agar; *the sequence identity is based on either forward or reverse primer.

The initial value of TVBN (30.1 mg/100 g) found in the present work in *P. notialis* is similar to those reported earlier in other crustaceans. Zeng et al. (2005) reported an initial value of 33.5 mg/100 g in shrimp (*Pandalus borealis*). Lopez-Caballero et al. (2007) found an initial value of 30 mg/100 g in pink shrimp (*Parapenaeus longirostris*). An initial value of 34 mg/100 g has been reported by Chinivasagam et al. (1996) in *Penaeus merguiensis*. However, lower initial values of TVBN in shrimps have also been documented. Mendes et al. (2002) found an initial value of 26 mg/100 g in *P. longirostris*. Chinivasagam et al. (1996) found an initial value of 22 mg/100 g in *Penaeus plebejus*. A value as low as 2 mg/100 g has been reported in Chinese shrimp (*Fenneropenaeus chinensis*) (Lu, 2009). TVBN values depend on fish species and are related to the fish non-protein nitrogen content, which in turn depends on type of fish feeding; catching season and region; fish size, age, and sex as well as microbial activity (Goulas and Kontominas, 2007; Kilinc and Cakli, 2005). TVBN values may also depend on the methods used for their determination (Botta et al., 1984). Therefore, standard rejection values cannot be applied to all species of shrimp (Chinivasagam et al., 1996). Mendes et al. (2002) suggested a limit value of 60 mg/100 g for pink shrimp (*P. longirostris*). In the present work, values of shrimp at the sensory rejection times (88-104 mg/100 g) suggest that a TVBN limit for *P. notialis* caught in tropical brackish waters may even be higher than 60 mg/100 g.

In the present work, TVBN seems to be a good indicator of shrimp spoilage. A good correlation was observed between TVBN values and sensory scores even during storage at 0ºC where TVC counts were lower at sensory rejection time compared to TVC counts at 28ºC and 7ºC. Evaluating the effect of temperature on bacterial load and microbiota composition in Norway lobster tail meat during storage, Gornik et al. (2011) also found that at sensory rejection time, the TVC counts were lower at 0ºC than the TVC counts at other storage temperatures (4-16ºC). The authors hypothesized that although in low number, the microorganisms had more time (sensory rejection time at 0ºC longer than sensory rejection times at other storage temperatures) to produce metabolites that were accumulated over the storage period. The increase in TVBN levels at 0ºC while the TVC counts were relatively low could also be explained by the fact that in addition to microbial spoilage, shrimp tissue enzymes also contribute to the production of volatile bases (Lopez-Caballaro et al., 2007; Matches, 1982; Mendes et al., 2002).

The initial value of TMA in the present study was approximately twelve times lower than the initial TVBN value. A low level of TMA in comparison with TVBN is in agreement with earlier observations. It has been reported that the main component of TVBN in crustaceans is ammonia (Vanderzant et al., 1973; Yeh et al., 1978). The acceptability limit of TMA in shrimp is 5 mg/100 g according to Cobb et al. (1973). However, as for TVBN, this limit should not be applied for all species of shrimp. In this study, the TMA values at sensory rejections times ranged between 9 and 12 mg/100 g. These values are similar to the value of 11.4 mg/100 g as the limit value of TMA for acceptable pink shrimp (Mendes et al., 2005).

The initial value of pH (7.4) is comparable to the initial values reported in other shrimp species by Goswami et al. (2001) (7.2), Lopez-Caballero et al. (2007) (7.3), Mendes et al. (2002) (7.1), and Zeng et al. (2005) (7.41). Like TVBN, pH increase during shrimp storage is due to enzymatic actions both endogenous and microbial which result in basic compounds production (Lopez-Caballaro et al. 2007). Our findings are in agreement with the report of Goswami et al. (2001), that the pH of shrimp is a fairly good indicator of its quality.

To assess the spoilage potential of microorganisms i.e. their ability to produce sensory defects, various sterile seafood substrates have been documented including sterile muscle blocks from a freshly killed seafood, ionized seafood, and heat-sterilized (100ºC, 30 min) seafood juice (Gram and Huss, 1996; Joffraud et al., 1998). Each of these treatments may fail to reproduce exactly the untreated product due to modification of the physico-chemical composition of the product (Gram and Huss, 1996; Leroi et al., 1998; Moini et al., 2009). Although heat-sterilization of seafood juice results in loss of amino acids (Leroi et al., 1998), heat-sterilization of whole shrimps seems to have less impact on their proximate composition. Delfieh et al. (2013) did not find any significant difference in protein and ash contents of raw and boiled (100ºC, 6 min) shrimp. In a comparative study of the spoilage of raw and boiled shrimps stored in 2ºC, Mendes et al. (2002) showed that although the

cooking step reduced the concentration of spoilage bacteria to below the detection limit in the beginning of storage, the microbiological and sensory spoilage were similar in both types of shrimps toward the end of storage (10-16 days).

The three temperatures used to develop the shelf-life model cover temperature variation range in the supply chain (from 0ºC (ice) to ambient temperature (28ºC)). Moreover, it has been demonstrated that three temperatures can be used to develop a shelf-life model prediction (Hough et al., 2006). The apparent activation energy estimated in this study (80.2±4.5 kJ mol^{-1}) is in concordance with previous values found with fresh food from tropical water (Dalgaard and Huss, 1997). As mentioned earlier by Dalgaard and Jorgensen (2000), mathematical models used to predict the shelf-life of seafood products may generate important differences depending on the types of seafood products. The common model (Eq. (4.6)) (based on Ratkowsky model) for fresh seafood shelf-life prediction (Dalgaard and Huss, 1997) predicts a shelf-life of 16.5 h for a shelf-life of 11 d at 0ºC (the average found in our study) and a storage temperature of 30ºC.

$$\text{Shelf life}(T) = \frac{\text{Shelf life}(T_0)}{(1+0.1\times T)^2} \tag{4.6}$$

with T, the temperature (ºC).

This prediction is unrealistic. The equation (4.6) fails to accurately predict the shelf-life when temperature abuse occurs because dominant spoilage flora shift from low temperature to high temperature (Dalgaard and Huss, 1997; Dalgaard and Jorgensen, 2000). An exponential model (Eq. (4.7)) for tropical fresh seafood shelf-life prediction has been proposed by Dalgaard and Huss (1997).

$$\text{Shelf life}(T) = \text{Shelf life}(T_0)\times\exp(-0.12\times T) \tag{4.7}$$

with T, the temperature (ºC).

This model gives predictions similar to the developed model in this study, especially at high storage temperature. For example, at 30ºC, the generated model in this study (Eq. (4.5)) predicts a shelf-life of 7.2 h. At the same temperature (30ºC) and for a shelf-life of 11 d at 0ºC, the model by Dalgaard and Huss (1997) (Eq. (4.7)) gives exactly the same prediction of 7.2 h. However, at 3ºC, the model generated in this study predicts a shelf-life of 6.8 days, while the model by Dalgaard and Huss (1997) predicts a shelf-life of almost one day longer (7.7 days).

In conclusion, the present work shows that the spoilage associations of shrimp (*P. notialis*) caught from brackish waters in Benin are storage temperature-dependent. During storage in ice (0ºC), *Pseudomonas* spp. were dominant, whereas at 7ºC and 28ºC, H_2S-producing bacteria were the dominant group of microorganisms. The potential spoilage organisms identified at 0ºC were *Pseudomonas* spp. and LAB. At 7ºC and 28ºC, LAB and *Enterobacteriaceae* were the main potential spoilage organisms. Good correlations were found between the sensory scores and the chemical indicators measured especially with total volatile basic nitrogen (TVBN) and trimethylamine (TMA). Therefore, TVBN or TMA can be used as an indicator of *P. notialis* quality. Modeling the spoilage kinetics of the relevant spoilage organisms in future studies will facilitate advising the stakeholders on the points in the supply chain that require special care to improve shrimp quality.

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Supplementary materials

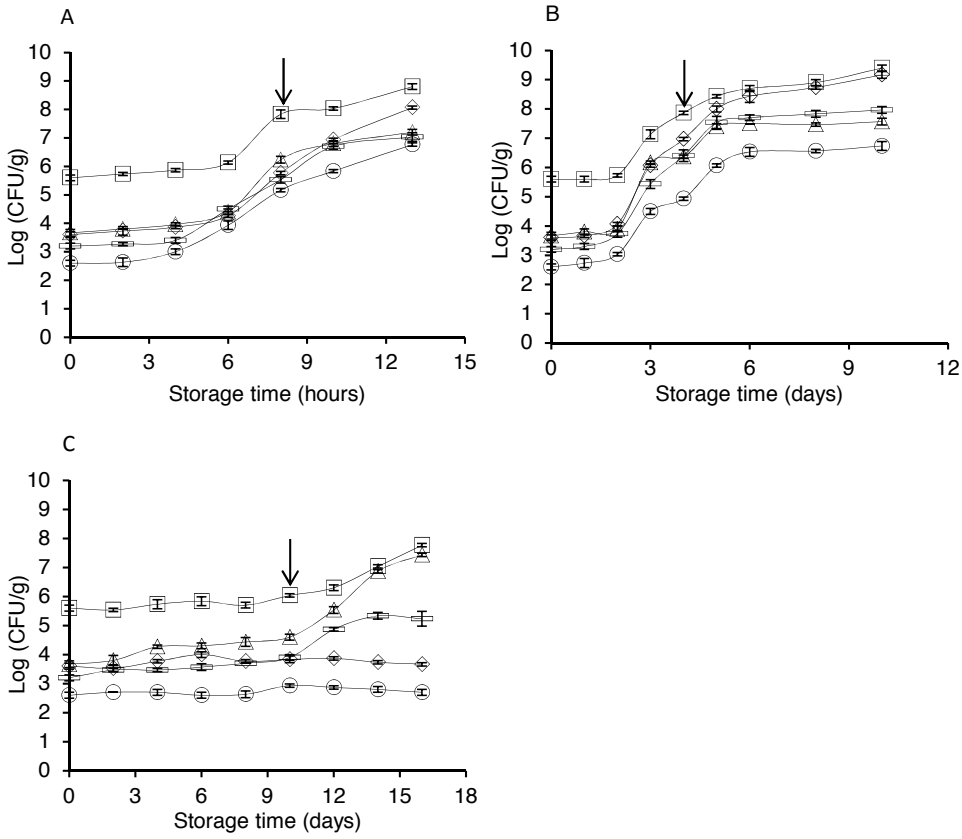

Fig. S4.1. Evolution of total viable counts (□), H_2S-producing bacteria (◇), *Pseudomonas* spp. (△), Enterobacteriaceae (○), and lactic acid bacteria (▭) of shrimp from lake Nokoue stored at 28ºC (A), 7ºC (B), and 0ºC (C). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

Table S4.1

Shrimp (from lake Nokoue) off-odor evaluation (in % of panelists) during storage at 28ºC (A), 7ºC (B), and 0ºC (C)

A

Odor evaluation	Storage time (hours)						
	0	2	4	6	8	10	13
No off-odor	90.0	80.0	60.0	13.3	3.3	0	0
Slight off-odor	10.0	20.0	40.0	76.7	46.7	26.7	0
Strong off-odor	0	0	0	10.0	**50.0**	73.3	100

B

Odor evaluation	Storage time (days)								
	0	1	2	3	4	5	6	8	10
No off-odor	90.0	83.3	63.3	0	0	0	0	0	0
Slight off-odor	10.0	16.7	36.7	53.3	20.0	6.7	0	0	0
Strong off-odor	0	0	0	46.7	**80.0**	93.3	100	100	100

C

Odor evaluation	Storage time (days)								
	0	2	4	6	8	10	12	14	16
No off-odor	90.0	76.7	76.7	10.0	3.3	3.3	0	0	0
Slight off-odor	10.0	23.3	23.3	90.0	80.0	46.7	50.0	40.0	30.0
Strong off-odor	0	0	0	0	16.7	**50.0**	50.0	60.0	70.0

Values in boldface indicate sensory rejection times.

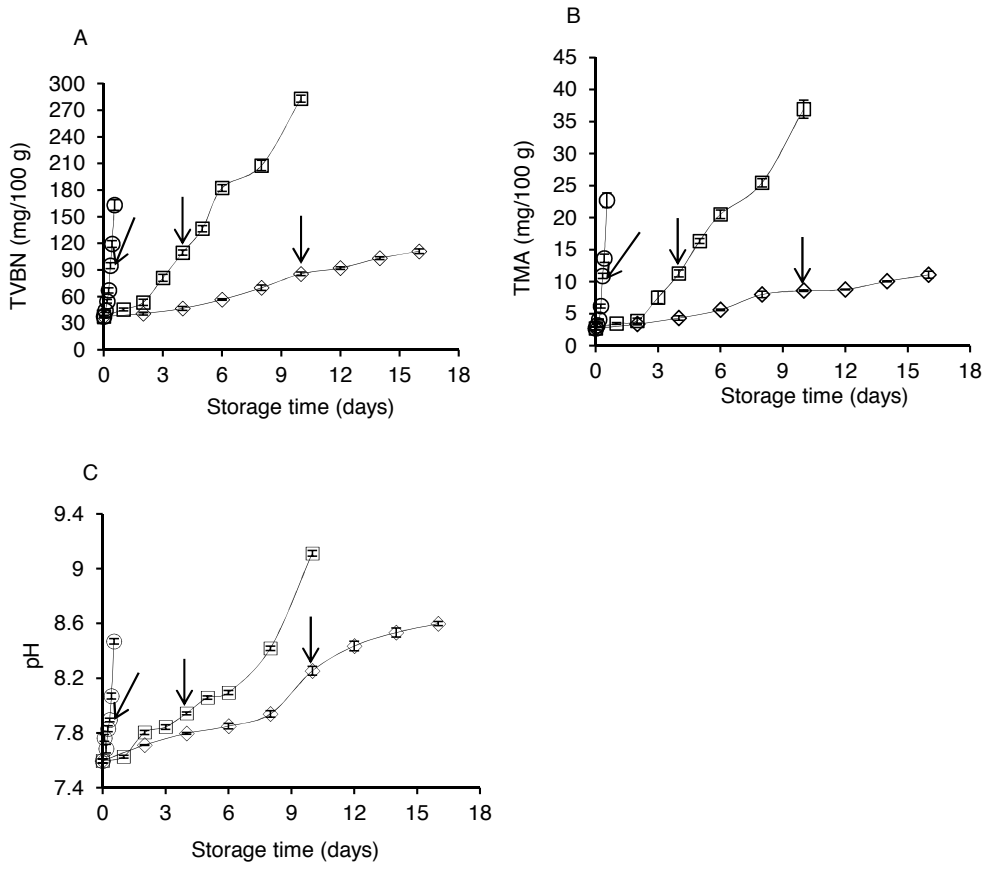

Fig. S4.2 Evolution of total volatile basic nitrogen (TVBN) (A), trimethylamine (TMA) (B), pH (C) of shrimp from lake Nokoue stored at 28ºC (○), 7ºC (□), and 0ºC (◇). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

5

Prediction of spoilage of tropical shrimp (*Penaeus notialis*) under dynamic temperature regimes

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Abstract

The spoilage activity of Pseudomonas psychrophila *and* Carnobacterium maltaromaticum*, two tropical shrimp (*Penaeus notialis*) spoilage organisms, was assessed in cooked shrimps stored at 0 to 28ºC. Microbiological, chemical and sensory analyses were performed during storage.* P. psychrophila *had a higher growth rate and showed a higher spoilage activity at temperatures from 0 to 15ºC, while at 28ºC,* C. maltaromaticum *had a higher growth rate. The spoilage activity of* P. psychrophila *was found to be higher in cooked shrimp than in fresh shrimp. Observed shelf-life data of shrimps stored at constant temperatures were used to validate a previously developed model that predicts tropical shrimp shelf-life at constant storage temperatures. Models predicting the growth of the spoilage organisms as a function of temperature were constructed. The validation of these models under dynamic storage temperatures simulating temperature fluctuation in the shrimp supply chain showed that they can be used to predict the shelf-life of cooked and fresh tropical shrimps.*

Introduction

Microbial spoilage of fishery products may result in high food losses (Gram and Dalgaard, 2002). Shrimps are particularly known as highly perishable products because, unlike other crustaceans (crabs, lobsters), which can be kept alive until processing, shrimps die soon after being caught (Adams and Moss, 2000). The possibility of estimating the residual shelf-life at any stage of a food supply chain is of importance to improve food quality management (Bruckner et al., 2013; Raab et al., 2008). To this end, quantitative microbiology can be an important tool and mathematical models can predict the progression of spoilage processes in foods (McMeekin and Ross, 1996). It is known that only a few members of the spoilage microflora, the so-called specific spoilage organisms (SSOs) produce the defects associated with fishery products spoilage (Dalgaard, 1995a; Gram and Dalgaard, 2002). Therefore, identification of SSOs and determination of their spoilage domain and spoilage level are prerequisites to accurately predict shelf-life (Dalgaard, 1995b; Koutsoumanis and Nychas, 2000). In a previous study, assessing the spoilage potential of bacteria isolated from fresh shrimps at the sensory rejection times, we found that isolates capable of producing strong off-odor in iced tropical brackish water shrimp (*Penaeus notialis*) were *Pseudomonas* spp. and psychrotrophic lactic acid bacteria (LAB) (Dabadé et al., 2015). However, the quantitative ability of the potential spoilage-causing organisms to spoil shrimps i.e. their spoilage activity was not assessed.

In the present work, the fastest growing isolates of each group, namely, *Pseudomonas psychrophila* and *Carnobacterium maltaromaticum* (LAB) were selected to assess their spoilage activity and to model their kinetics during shrimp shelf-life. Models on *Pseudomonas* spp. and *C. maltaromaticum* have been reported in the literature. Examples are models developed by Alfaro et al. (2013), Bruckner et al. (2013), Gospavic et al. (2008), Koutsoumanis (2001), Lebert et al. (1998), and Neumeyer et al. (1997). However, some of these models were developed using media that do not take into account a potential food matrix effect. Moreover, most of the models were developed in a range of temperatures that are not representative for tropical regions. For instance, the predictive model for the growth of *C. maltaromaticum* by Alfaro et al. (2013) is not reliable above 20ºC according to the authors. Ambient temperatures in tropical areas are however often higher than 25ºC. Therefore, the objectives of the present work were to develop mathematical models for the growth of *P. psychrophila* and *C. maltaromaticum* in cooked shrimp (*Penaeus notialis*) from tropical brackish water that are stored at temperatures relevant for the shrimp supply chain, which ranges from 0 to 28ºC. A model describing the growth of *Pseudomonas* spp. in fresh shrimps as a function of temperature was also developed. Moreover, models were validated under non-isothermal conditions that reflect temperature fluctuation along the supply chain.

Materials and methods

Inocula preparation

P. psychrophila and *C. maltaromaticum* were pre-cultured in 10 ml of Brain Heart Infusion (BHI) broth (OXOID) for 2 days at 25ºC. The cultures were diluted in peptone physiological saline (pps) (0.85% NaCl (SIGMA), 0.1% peptone (OXOID)) in order to obtain a concentration of about 6 log CFU/ml.

Shrimp sampling preparation and storage

Shrimps used in the present study were collected from Lake Aheme, one of the most important shrimp fishing areas in Benin (West Africa). The weight of an individual shrimp was approximately 20 g. Immediately after being caught, shrimps were cooled with ice and transferred to the laboratory to arrive within 2 h. In the laboratory, shrimp samples were divided in two batches. One batch was kept fresh whereas the other batch was cooked (100ºC, 30 min). Cooked and fresh shrimp samples were aseptically packed in sealed sterile polyethylene bags (Twirl'em sample bags, Labplas Inc, dimensions: 305×178 mm; thickness: 89 μm). Each pack of cooked shrimp (approximately 300 g shrimp) was inoculated with 3 mL of prepared *P. psychrophila* inoculum or *C. maltaromaticum* inoculum to obtain an initial concentration of about 4 log CFU/g. Non-inoculated packs of

cooked shrimps in which total viable counts were below the detection limit (1 log CFU/g) (data not shown) were used as control. Packs of fresh shrimps were inoculated as described for cooked shrimps, but only with *P. psychrophila* inoculum. Also for fresh shrimp non-inoculated packs were used as control. Shrimps were stored at 0ºC, 4ºC, 7ºC, 15ºC, and 28ºC as previously described (Dabadé et al., 2015). At appropriate time intervals, shrimp samples were aseptically taken out from the packs for microbiological, chemical and sensory analyses. Three packs of shrimps were used to obtain triplicate analysis per sampling time point.

Microbiological analysis

Whole shrimp (25 g) and 225 mL of pps were transferred aseptically into a stomacher bag. The mixture was homogenized for 60 s using a stomacher (Seward Laboratory Stomacher 400, England). From this first decimal dilution in pps, appropriate decimal dilutions were prepared. *Pseudomonas* species were enumerated on spread plates of *Pseudomonas* agar base (OXOID) supplemented with cetrimide, fucidin, and cephaloridine (CFC) (OXOID) and the plates were incubated at 25°C for 48 h.

C. maltaromaticum was enumerated on iron agar (IA) supplemented with 0.04% L-cysteine (SIGMA) and the plates were incubated at 25ºC for 72 h as described by Gram et al. (1987).

Chemical analysis

The method recommended by the European Commission (Commission regulation (EC) No 2074/2005) was used to determine total volatile basic nitrogen (TVBN) in cooked and fresh shrimp. Briefly, perchloric acid (SIGMA) shrimp extract was steam-distilled and the volatile base components were determined by titration.

Sensory analysis

The odor and texture of cooked shrimp, and the odor, color and texture of fresh shrimp were evaluated individually by ten panelists experienced in shrimp freshness evaluation. The overall acceptance of the shrimps was assessed using a scale with three categories: 1= shrimp with good quality, 2= shrimp with marginal quality, but still acceptable 3 = spoiled shrimp (Argyri et al., 2010; Dalgaard et al., 1993; Mejlholm et al., 2005). Sensory rejection time was defined as the moment when 50% of the panelists evaluated samples to be in category 3. When the product was rejected with more than 50% of the panelists on a sampling date, then the rejection time was estimated by interpolation.

Statistical analysis

A Student's two-tailed *t*-test or one-way ANOVA (IMB SPSS Statistics 19.0) followed by Tukey's test as post hoc comparison of means was used to compare the means of data (log counts, TVBN, maximum specific growth rate) from different types of shrimps. Significance was set at $P < 0.05$.

Primary modeling

The Baranyi model (Baranyi and Roberts, 1994) as described by Den Besten et al. (2006) (Eq. (5.1)) and the reparameterized Gompertz model (Zwietering et al., 1990) (Eq. (5.2)) were fitted to the microbial growth data obtained at 0, 4, 7, 15, and 28ºC using Excel's solver function and verified with DMFit curve-fitting software v2.1 (http://www.ifr.ac.uk/safety/DMFit/default.html).

$$\log_{10}(N_t) = \log_{10}(N_0) + \frac{\mu_{max}}{\ln(10)} \cdot A(t) - \frac{1}{\ln(10)} \cdot \ln\left[1 + \frac{\exp(\mu_{max} \cdot A(t)) - 1}{10^{(\log_{10}(N_{max}) - \log_{10}(N_0))}}\right] \quad (5.1)$$

where *A(t)* is an adjustment function described by Baranyi and Roberts (1994), *t* is the time (h), N_t is the number of microorganisms at time *t* (CFU/g), N_0 is the number of microorganisms at time zero (CFU/g), N_{max} is the maximum number of microorganisms (CFU/g), μ_{max} is the maximum specific growth rate (per hour).

$$\log_{10}(N_t) = \log_{10}(N_0) + [\log_{10}(N_{\max}) - \log_{10}(N_0)] \exp\left\{-\exp\left[\frac{\frac{\mu_{\max}}{\ln(10)} \cdot \exp(1)}{[\log_{10}(N_{\max}) - \log_{10}(N_0)]}(\lambda - t) + 1\right]\right\} \quad (5.2)$$

where t is the time (h), N_t is the number of microorganisms at time t (CFU/g), N_t is the number of microorganisms at time zero (CFU/g), $N_{\max}$ is the maximum number of microorganisms (CFU/g), $\mu_{\max}$ is the maximum specific growth rate (per hour), and λ is the lag time (h).

Secondary modeling

To model the effect of temperature on microbial growth, two equations were used: the Ratkowsky equation (Eq. (5.3)) (Ratkowsky et al., 1982) and the Arrhenius equation (Eq. (5.4)).

$$\mu_{\max} = [b_1(T - T_{\min})]^2 \quad (5.3)$$

$$\mu_{\max} = b_2\left[\exp\left(\frac{-E_a}{R\theta}\right)\right] \quad (5.4)$$

where $\mu_{\max}$ is the maximum specific growth rate (per hour), T is temperature (in degrees Celsius), θ is temperature (in degrees Kelvin), R is the gas constant (8.314 J mol^{-1} K^{-1}), b_i, $T_{\min}$, and E_a are the regression coefficients to be estimated. $T_{\min}$ (in degrees Celsius) is the extrapolated temperature at which a microorganism cannot grow anymore. E_a (in J mol^{-1}) is the activation energy.

Comparison of model

To assess the performance of the two secondary models used, we compared the root mean square error of the models ($RMSE_{\text{model}}$) (Eq. (5.5)) as described by Den Besten et al. (2006) after both ln-transformation and square-root-transformation of the data. The fitting of the models was performed on the same scale as the data transformation.

$$RMSE_{\text{model}} = \sqrt{\frac{\sum(\text{observed}_i - \text{fitted}_i)^2}{n - s}} \quad (5.5)$$

with observed_i are observed values, fitted_i are described values, n is the number of data points, s is the number of parameters of the model.

Validation of model

Two types of models were validated in this study. First, a model (Eq. (5.6)) that predicts the shelf-life of fresh tropical shrimp at any constant storage temperature and which was developed in a previous study (Dabadé et al., 2015).

$$\text{Shelf-life (days)} = 4.5\times10^{-15} \times \exp\left(\frac{9650}{\theta}\right) \quad (5.6)$$

where θ is the storage temperature in degrees Kelvin. The model was validated in this study using observed shelf-life data of shrimps stored at constant temperatures. Second, independent experiments were conducted to validate the models developed in this study, that predict the growth of *P. psychrophila* in cooked shrimps and *Pseudomonas* spp. in fresh samples as function of temperature. Shrimps were collected and were stored under dynamic temperatures using two scenarios that simulated the temperature fluctuation in the field. During the first scenario, shrimps were stored first at ambient temperature (28°C) for 6 h, after which they were stored alternating 12 h at 10°C and 12 h at 5°C (scenario 28/10/5°C). During the second scenario, caught shrimp were stored alternating 24 h in ice (0°C) and 24 h at 7°C (scenario 0/7°C). The first scenario is based on the most common shrimp collection and transportation system in which after being caught, shrimps are kept up to 6 h at

ambient temperature (28°C) before their storage in ice using non-isothermal containers. Shrimps are not always properly iced and their temperature may reach 10°C at the reception in shrimp plants (Dabadé et al., 2014). The second scenario is based on a new system of shrimp collection and transportation that is being promoted. In this new system, freshly caught shrimps are stored in ice by fishermen as soon as possible and collected shrimps are transported to shrimp plants using isothermal containers. Shrimp temperature should not exceed 7°C at any point of the chain in the new system. To predict microbial growth under non-isothermal conditions, a three phase model (Eq. (5.7)) (Buchanan et al., 1997) taking into account the maximum specific growth rate estimated with the Ratkowsky equation was used. The average of the respective initial and maximum microbial concentrations obtained during the storage experiments at constant temperature was used.

$$\begin{aligned} &\log_{10}(N_t) = \log_{10}(N_0) \text{ for } t < \lambda \\ &\log_{10}(N_t) = \log_{10}(N_0) + \frac{\mu_{\max}}{\ln(10)} \cdot (t - \lambda) \text{ for } \lambda < t < t_{\max} \\ &\log_{10}(N_t) = \log_{10}(N_{\max}) \text{ for } t \geq t_{\max} \end{aligned} \quad (5.7)$$

where t is time (h), N_t is the number of microorganisms at time t (CFU/g), N_0 is the number of microorganisms at time zero (CFU/g), λ is the lag time (h), $t_{\max}$ is the time when the maximum number of microorganisms is reached (h), $\mu_{\max}$ is the maximum specific growth rate (per hour), $N_{\max}$ is the maximum number of microorganisms (CFU/g).

It has been shown that the maximum specific growth rate ($\mu_{\max}$) is reciprocally proportional to the lag time (λ) (Baranyi and Roberts, 1994; Koutsoumanis et al., 2006; McMeekin et al., 1993; Zwietering et al., 1994a). Although the product $\mu_{\max} \times \lambda$ is not always constant (Koseki and Nonaka, 2012), it generally ranges between approximately 0 and 4 (Koutsoumanis et al., 2006; Zwietering et al., 1994a). In the present study, the lag time was estimated assuming that $\mu_{\max} \times \lambda = 1$ and $\mu_{\max} \times \lambda = 4$. It was also assumed that there is no additional lag time when a temperature shift occurred during the exponential phase and a temperature shift during the lag phase resulted in a new lag time equivalent to the relative part of the remaining lag phase (Zwietering et al., 1994b).

The performance of the developed models was assessed by graphical comparison of predicted and observed values. In addition, the bias (Eq. (5.8)) and the accuracy (Eq. (5.9)) factors according to Ross (1996) were calculated to assess the performance of the developed models as follows:

$$B_f = 10^{\left(\sum \log\left(t_{predicted}/t_{observed}\right)/n\right)} \quad (5.8)$$

$$A_f = 10^{\left(\sum \left|\log\left(t_{predicted}/t_{observed}\right)\right|/n\right)} \quad (5.9)$$

In case of the validation of the model that predicts the shelf-life of fresh tropical shrimp at constant storage temperatures, $t_{predicted}$ are the predicted shelf-life values (in days), $t_{observed}$ are the observed shelf-life values (in days). In the case of the validation of the models that predict the growth of pseudomonads in shrimps under dynamic temperature regimes, $t_{observed}$ are times (in hours) at which a given pseudomonads level was observed, $t_{predicted}$ are predicted times (in hours) to reach this same pseudomonads level. In both cases, n is the number of observations. In case of the validation of the model predicting shelf-life at constant temperature, B_f values higher than 1 show that the predicted shelf-life is on average longer than the observed shelf-life. In case of the validation of the model predicting the growth of pseudomonads, B_f values higher than 1 show that the predicted time to reach the concentration of pseudomonads that indicates spoilage is on average longer than the observed time to reach that concentration. B_f = 1 suggests, on average, a perfect agreement between predicted and observed values. The accuracy factor assesses the absolute deviation between the observed values and the predicted values. A_f = 1 shows a perfect agreement between predicted and observed values. The larger the accuracy factor, the less accurate is the average estimate (Ross, 1996).

The performance of the model predicting shrimp shelf-life at constant storage temperature was also evaluated using the acceptable prediction zone method developed by Oscar (2005). The relative errors (RE) for shelf-life was calculated

$$RE = (\text{predicted} - \text{observed})/\text{predicted} \quad (5.10)$$

RE less than zero represents fail-safe predictions and RE above zero represents fail-dangerous predictions (Oscar, 2005). The boundaries of the acceptable prediction zone proposed by Oscar (2005), which were -0.3 (fail-safe) and 0.15 (fail-dangerous), were used.

Results

Microbial growth during shrimp storage

Of the two primary models used to describe microbial growth as function of time, the root mean square error of the reparameterized Gompertz model was in general lower than the root mean square errors of the Baranyi model (data not shown). Therefore, the reparameterized Gompertz model was used in this study to describe microbial growth data. The microbial growth data of the different shrimp products with the fitted reparameterized Gompertz model are shown in Fig. 5.1. In cooked inoculated shrimps (Fig. 5.1A and 5.1B), the initial microbial concentration (ca. 4 log CFU/g) increased as expected with a higher rate at higher storage temperatures to maximum concentrations of approximately 10 log CFU/g at most of the storage temperatures. The initial *Pseudomonas* spp. concentrations were 4.1±0.2 log CFU/g (mean ± standard deviation) in fresh shrimp inoculated with *P. psychrophila* (Fig. 5.1C) and 3.8±0.2 log CFU/g in fresh non-inoculated shrimp (Fig. 5.1D). In fresh shrimps, the inoculated samples had a shorter lag phase than the non-inoculated. While in fresh non-inoculated shrimp, the maximum concentration of *Pseudomonas* spp. reached 9.1±0.1 log CFU/g at low storage temperatures (0ºC and 4ºC), the maximum concentrations were approximately 7.8±0.1 log CFU/g at 7 and 15ºC and 6.9±0.2 log CFU/g at 28ºC. Fresh shrimps inoculated with *P. psychrophila* also showed a maximum concentration of *Pseudomonas* spp. of 7.1±0.1 log CFU/g at 28ºC. However, the maximum concentration reached 9.3±0.1 log CFU/g at 7ºC and 8.4±0.2 log CFU/g at 15ºC. High correlation (R^2 >0.90) was found between the mean concentration of *P. psychrophila* in cooked shrimps and the mean concentration of *Peudomonas* spp. in fresh shrimps inoculated with *P. psychrophila* at storage temperatures ranging between 0ºC and 15ºC. At 28ºC, a lower correlation was found (R^2 = 0.73) (Fig. S5.1).

In general, a high concentration (8-9 log CFU/g) of the inoculated bacteria in cooked shrimps was obtained at the sensory rejection times, while the microbial concentration of *Pseudomonas* spp. in fresh shrimps at the sensory rejection times was lower.

Total volatile basic nitrogen production (TVBN) during storage

Changes in TVBN values of the different shrimp samples are shown in Fig. 5.2. The initial TVBN value in freshly caught shrimp was 29.2±2 mg/ 100 g. The cooking step lowered significantly the initial TVBN value to an average of 10.9±1.5 mg/ 100 g in cooked shrimps. As expected, the TVBN values increased with time and temperature during the storage of the different shrimp samples. The TVBN values of cooked shrimps inoculated with *P. psychrophila* were significantly higher than the TVBN values of cooked shrimps inoculated with *C. maltaromaticum* during storage at temperatures ranging from 0 to 15ºC. At 28ºC however, no significant difference in the TVBN values was obtained. The TVBN values in fresh shrimps were significantly higher compared to cooked shrimps until the end of storage. The TVBN values at the sensory rejection times in cooked shrimps ranged between 43 and 54 mg/100 g. Higher values were found with fresh shrimps (73-85 mg/100 g).

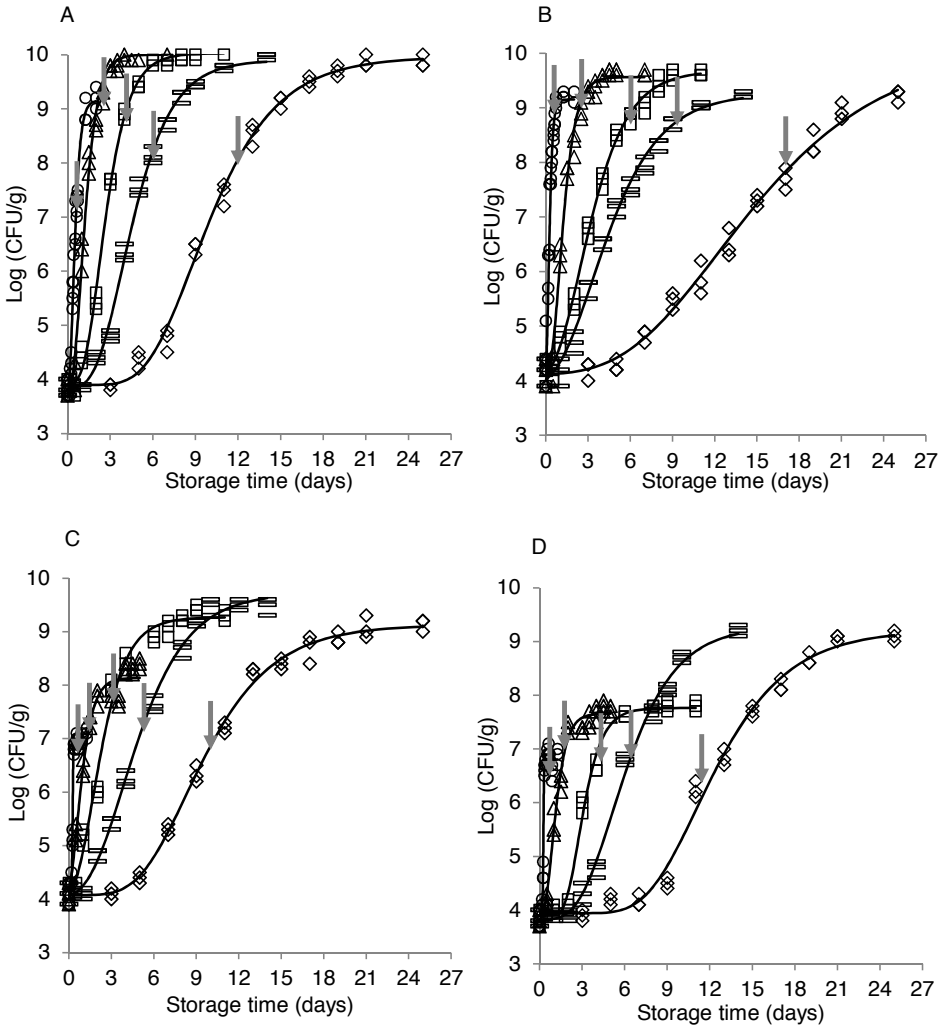

Fig. 5.1 Evolution of *Pseudomonas psychrophila* in cooked shrimps (A), *Carnobacterium maltaromaticum* in cooked shrimps (B), *Pseudomonas* spp. in fresh shrimps inoculated with *Pseudomonas psychrophila* (C), *Pseudomonas* spp. in fresh non-inoculated shrimps (D) during storage at 0ºC (◇), 4ºC (▭), 7ºC (□), 15ºC (△), and 28ºC (○). The reparameterized Gompertz model (solid line) was fitted to the growth data. Arrow indicates sensory rejection time.

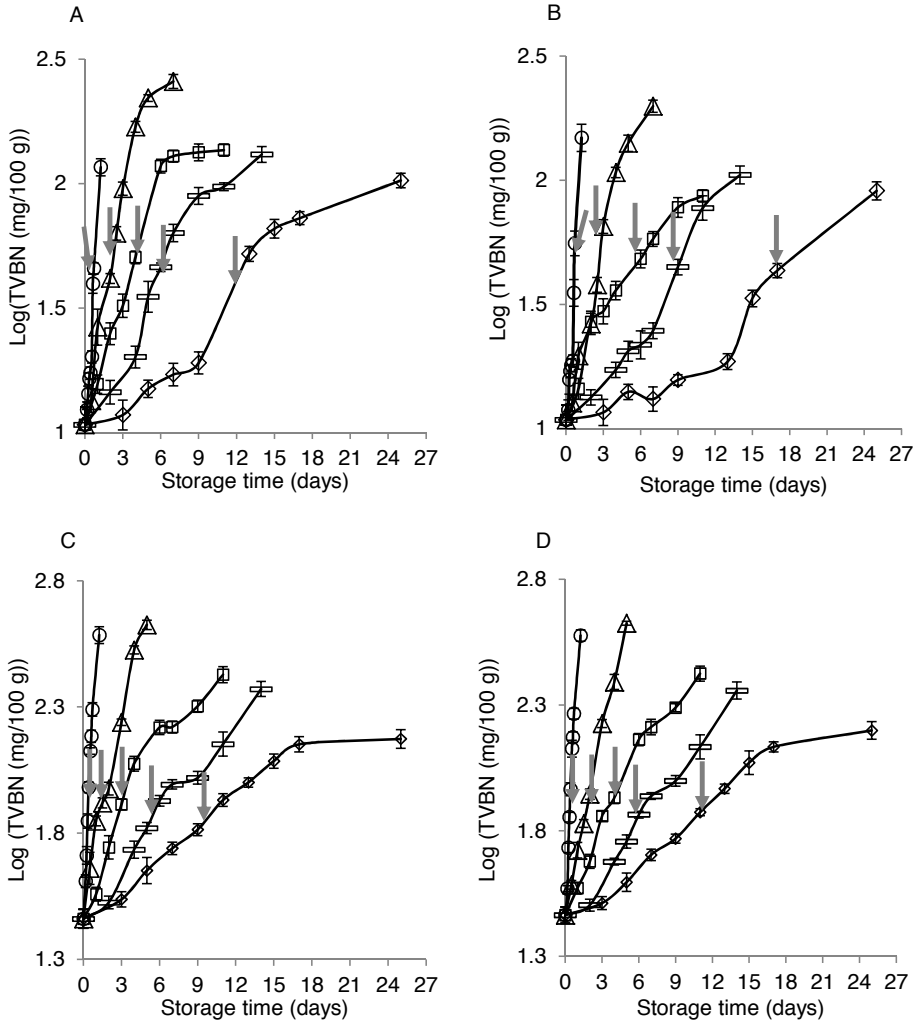

Fig. 5.2 Evolution of total volatile basic nitrogen (TVBN) in cooked shrimps inoculated with *Pseudomonas psychrophila* (A), cooked shrimps inoculated with *Carnobacterium maltaromaticum* (B), fresh shrimps inoculated with *Pseudomonas psychrophila* (C), fresh non-inoculated shrimps (D) during storage at 0ºC (◇), 4ºC (▭), 7ºC (□), 15ºC (△), and 28ºC (○). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

Modeling the maximum growth rate as a function of temperature

Of the two secondary models used (Eqs. (5.3) and (5.4)), the Ratkowsky model (Eq. (5.3)) had the lower root mean square error of the residuals ($RMSE_{model}$) (data not shown) and fitted better to the maximum specific growth data as shown in Fig. 5.3 with *P. psychrophila* in cooked shrimp. The residuals plot of both models is also depicted in Fig. S5.2 for graphical comparison. Therefore, the Ratkowsky equation was used in this study as secondary model, and the estimated parameters are shown in Table 5.1. The effect of temperature on the maximum specific growth rate of *P. psychrophila* and *C. maltaromaticum* in cooked shrimps is shown in Fig. 5.4A. The growth rate of *P. psychrophila* in cooked shrimp was significantly higher than the growth rate of *C. maltaromaticum* in cooked shrimp at temperatures from 0 to 15ºC. At 28ºC, however, the growth rate of *C.*

maltaromaticum was significantly higher than the growth rate of *P. psychrophila*. In fresh shrimps, the growth rates of *Pseudomonas* spp. in inoculated and non-inoculated samples were similar (data not shown).

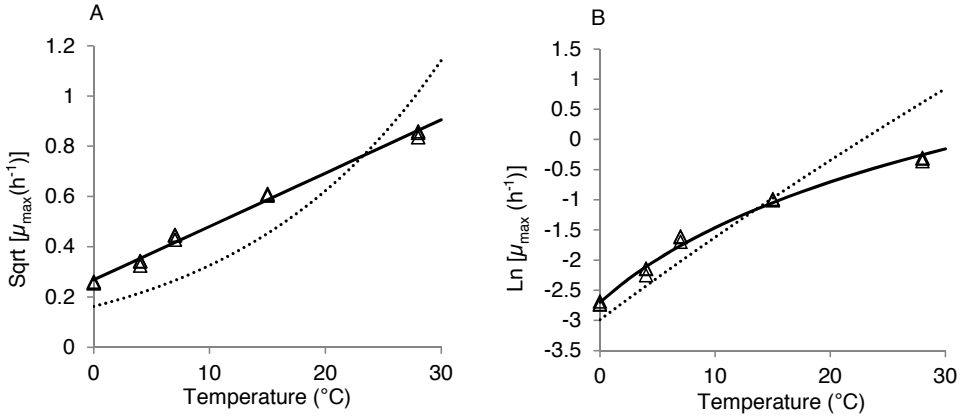

Fig. 5.3 Maximum specific growth rate of *Pseudomonas psychrophila* in cooked shrimp at square root scale (A) and ln scale (B) as function of temperature modelled by the Ratkowsky equation (solid line) and Arrhenius equation (dotted line). Both models are represented and fitted on square root scale (A) and ln scale (B).

Table 5.1

Parameters (with 95% C.I.) of the temperature growth rate dependency estimated with the Ratkowsky equation (Eq. (5.3))

Microorganisms	T_{min}	b_1
P. psychrophila in cooked shrimp	-12.6 (-13.4, -11.8)	0.021 (0.020, 0.022)
C. maltaromaticum in cooked shrimp	-4.2 (-5.3, -3.1)	0.032 (0.030, 0.034)
Pseudomonas spp. in fresh shrimps inoculated with *P. psychrophila*	-12.1 (-13.1, -11.1)	0.019 (0.017, 0.021)
Pseudomonas spp. in non-inoculated shrimps	-12.0 (-13. 1, -10.9)	0.019 (0.017, 0.021)

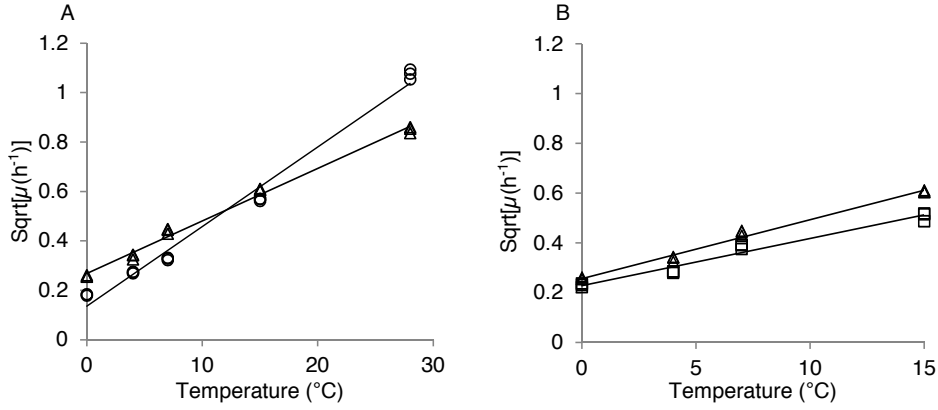

Fig. 5.4 Maximum specific growth rate of *Pseudomonas psychrophila* (Δ) and *Carnobacterium maltaromaticum* (○) in cooked shrimps (A), *Pseudomonas psychrophila* (Δ) in cooked shrimps and *Pseudomonas* spp. (□) in fresh non-inoculated shrimps (B) at different temperatures modelled by the Ratkowsky equation (solid line).

The growth rate of *P. psychrophila* in cooked shrimps was significantly higher than the growth rate of *Pseudomonas* spp. in fresh non-inoculated shrimps at temperatures ranging from 0 to 15ºC (Fig. 5.4B). At 28ºC, the growth rate was high and we could not accurately estimate the maximum growth rate of *Pseudomonas* spp. in fresh shrimps due to limited data points collected.

Validation of the model predicting shrimp shelf-life at constant storage temperatures

A graphical comparison of the predicted shrimp shelf-life values from an earlier study (Eq. (5.6)) and those experimentally observed at constant temperature in this study with different type of shrimps is shown in Fig. 5.5. Relative errors for shrimp shelf-life (days) (Eq. (5.10)) plot against storage temperatures of the different type of shrimps are also shown in Fig. S5.3. The bias factors and accuracy factors of the model are presented in Table 5.2. The B_f values ranged between 0.61 and 1.06 and the A_f , between 1.06 and 1.65 for the types of shrimps used.

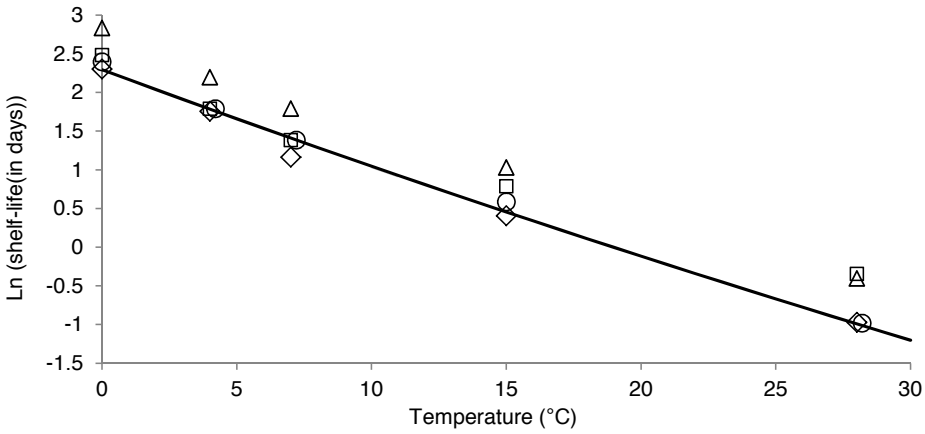

Fig. 5.5 Predicted fresh shrimps shelf-life (solid line) and observed shelf-lives of cooked shrimps inoculated with *Pseudomonas psychrophila* (□), cooked shrimps inoculated with *Carnobacterium maltaromaticum* (△), fresh shrimps inoculated with *Pseudomonas psychrophila* (◇), fresh non-inoculated shrimps (○).

Table 5.2

Bias factor and accuracy factor of the model predicting fresh shrimp shelf-life at constant storage temperatures

Types of shrimps	Bias factor (B_f)[a]	Accuracy factor (A_f)[a]
Cooked shrimp inoculated with *P. psychrophila*	0.79	1.27
Cooked shrimp inoculated with *C. maltaromaticum*	0.61	1.65
Fresh shrimp inoculated with *P. psychrophila*	1.06	1.08
Non-inoculated fresh shrimps	0.95	1.06

[a] B_f and A_f were calculated based on the comparison between the predicted and observed shelf-life.

Models validation and shrimp shelf-life prediction and validation under non-isothermal conditions

The predicted and observed microbial growth under non-isothermal conditions is depicted in Fig. 5.6. The bias factors and accuracy factors of the models are shown in Table 5.3. The models developed based on a low value of the product maximum specific growth rate and lag time ($\mu_{\max} \times \lambda = 1$) gave better prediction with the scenario involving high storage temperatures (scenario 28/10/5), while with the low temperatures scenario (0/7),

a high value of the product maximum specific growth rate and lag time ($\mu_{max} \times \lambda = 4$) was more appropriate for pseudomonads growth prediction.

Table 5.3

Bias factor and accuracy factor of the models predicting the growth of *Pseudomonas psychrophila* in cooked shrimps and *Pseudomonas* spp. in fresh shrimps at two temperature profiles

	Temperature profile[a]	Bias factor (B_f)[b]	Accuracy factor (A_f)[b]
P. psychrophila in cooked	28/10/5	0.77	1.32
shrimps	0/7	0.83	1.21
Pseudomonas spp. in fresh	28/10/5	0.90	1.26
shrimps	0/7	0.85	1.19

[a]28/10/5: shrimps were stored at 28°C for 6 h, then they were stored alternating 12 h at 10°C and 12 h at 5°C (see Fig. 5.6A or 6C)
0/7: shrimps were stored alternating 24 h at 0°C and 24 h at 7°C (see Fig. 5.6B or 5.6D)
[b]B_f and A_f are values of the models developed based on $\mu_{max} \times \lambda = 1$ for profile 28/10/5 and $\mu_{max} \times \lambda = 4$ for temperature profile 0/7. They were calculated based on the comparison between the times microbial counts were observed (enumerated) and the times predicted to reach the same concentration as that observed (enumerated).

Table 5.4

Predicted and observed shelf-life of cooked tropical shrimp (inoculated with *Pseudomonas psychrophila*) and naturally contaminated tropical shrimp under dynamic temperature storage

	Temperature profile[a]	Observed shelf-life (h)	Predicted shelf-life (h)	Difference[b] (%)
P. psychrophila in cooked	28/10/5	54	55	-1.9
shrimps	0/7	152	140.5	+7.6
Pseudomonas spp. in fresh	28/10/5	30	32	-6.7
shrimps	0/7	144	122	+15.3

[a]28/10/5: shrimps were stored at 28°C for 6 h, then they were stored alternating 12 h at 10°C and 12 h at 5°C (see Fig. 5.6A or 6C)
0/7: shrimps were stored alternating 24 h at 0°C and 24 h at 7°C (see Fig. 5.6B or 5.6D)
[b]Difference between observed and predicted shelf-life values.

The developed growth models were used to predict the shelf-life of shrimps at non-isothermal conditions. The shelf-life of cooked shrimps inoculated with *P. psychrophila* was defined as the time at which the microbial concentration is 8.5 log CFU/g, which was the average concentration of *P. psychrophila* at the sensory rejection time obtained in this study when shrimps were stored at constant temperatures. Fresh shrimp shelf-life was defined as the time at which the concentration of *Pseudomonas* spp. reaches 6.5 log CFU/g, which was the average concentration of *Pseudomonas* spp. in fresh non inoculated shrimps at the sensory rejection time obtained in this study when shrimps were stored at constant temperatures. The predicted shelf-life values based on the models and the observed shelf-life values by sensory analysis are shown in Table 5.4.

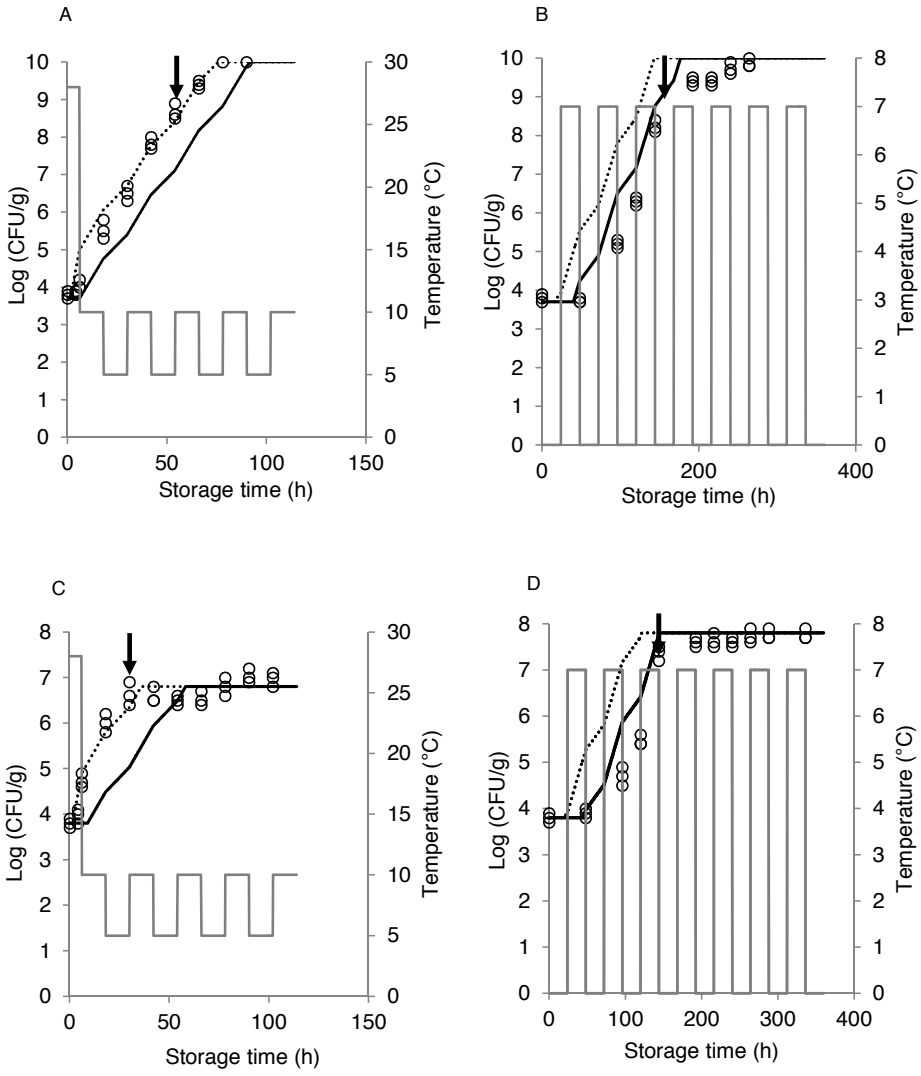

Fig. 5.6 Observed growth of spoilage organisms and predicted growth based on the assumption $\mu_{max} \times \lambda = 1$ (dotted line) and the assumption $\mu_{max} \times \lambda = 4$ (black solid line) at temperature profile 28/10/5ºC in cooked shrimp (A), and fresh shrimp (C) and temperature profile 0/7ºC in cooked shrimp (B), and fresh shrimp (D). The spoilage organisms are *Pseudomonas psychrophila* and *Pseudomonas* spp. in cooked and fresh shrimp, respectively. Grey solid line indicates temperature profile.
28/10/5ºC: shrimps were stored at 28°C for 6 h, then they were stored alternating 12 h at 10°C and 12 h at 5°C
0/7ºC: shrimps were stored alternating 24 h at 0°C and 24 h at 7°C. Arrow indicates observed sensory rejection time. To construct the models, it was assumed that there is no additional lag time when a temperature shift occurred during the exponential phase and a temperature shift during the lag phase resulted in a new lag time equivalent to the relative part of the remaining lag phase.

Discussion

The present study aimed to assess the spoilage activity of *P. psychrophila* and *C. maltaromaticum* in tropical shrimp (*Penaeus notialis*), to develop models that predict the growth of spoilage bacteria in *Penaeus notialis* as a function of temperature and to validate the models.

The ability of *P. psychrophila* and *C. maltaromaticum* to produce off-odor in shrimp has been reported (Dabadé et al. 2015). According to Gram et al. (2002), to identify the most important spoilage microorganisms of a product, it is crucial to assess their quantitative ability to produce spoilage metabolites. The present work shows that *P. psychrophila* produced significantly higher amounts of total volatile basic nitrogen (TVBN) than *C. maltaromaticum* at storage temperatures varying between 0-15ºC. This is consistent with the sensory analysis that showed a shorter rejection time with cooked shrimps inoculated with *P. psychrophila* in comparison with cooked shrimp inoculated with *C. maltaromaticum* between 0 and 15ºC. The chemical and sensory results agreed with the microbiological results since within the same temperature range, the growth rate of *P. psychrophila* was significantly higher than that of *C. maltaromaticum*. Although at 28ºC the growth rate of *C. maltaromaticum* was significantly higher than that of *P. psychrophila,* the sensory rejection times of shrimps inoculated with both organisms were comparable. Our microbiological, chemical and sensory results suggest that *P. psychrophila* spoils air-stored tropical shrimp (*Penaeus notialis*) faster than *C. maltaromaticum* at temperatures ranging from 0 up to 15ºC. Similar results have been reported in meat by Casaburi et al. (2011) and Ercolini et al. (2010) who showed that *Pseudomonas fragi* had a higher sensory impact on meat stored at 4ºC than *C. maltaromaticum* in air or vacuum-packaged. In contrast, Macé et al. (2014) showed that in cooked tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging at 8ºC, *C. maltaromaticum* was the strongest spoiler in comparison with other bacterial species including a member of *Pseudomonadales* (*Psychrobacte*r sp.). In our study, *C. maltaromaticum* as well as *P. psychrophila* were inoculated in shrimps of which the endogenous flora was heat inactivated. Thus, it is unlikely that the inoculated microorganisms competed for resources such as nutrients and oxygen with other microorganisms. Also, oxygen was not probably a limiting factor since packs of shrimps stored at 28°C were opened almost every 2 h for sampling, and every 24-48 h at the lower temperatures. Our data on spoilage of tropical shrimp (*Penaeus notialis*) are in agreement with recent studies on spoilage of cooked cold water shrimp (*Pandalus borealis*) (Laursen et al., 2006) and cooked tropical shrimp (*Penaeus vannamei*) (Jaffres et al., 2011; Macé et al., 2014) showing that *C. maltaromaticum* produces volatile compounds such as ammonia (TVBN compound) and other chemical compounds that spoil cooked shrimp. However, whether we should consider *C. maltaromaticum* as a weak or strong shrimp spoiler, will depend on the types of other spoilage flora associated with the product, which in turn depend on the storage conditions. *C. maltaromaticum*, which is known to show high resistance to CO_2 and to lack of O_2 (Alfaro et al., 2013), can outcompete aerobic bacteria and become the strongest spoiler in modified-atmosphere-packaging or vacuum-packaging, but in air-stored shrimp, its spoilage impact is limited in the presence of more active aerobic spoilers such as *Pseudomonas* spp.

The microbiological analysis revealed that *Pseudomonas* spp. were the predominant microorganisms in fresh shrimp at low storage temperatures (0 and 4ºC) with a maximum concentration reaching 9 log CFU/g. However, their maximum concentration was 1-2 log CFU/g lower at storage temperatures ranging from 7 to 28ºC. This difference could be explained by an antagonistic or competitive activity of other groups of microorganisms that predominate at high storage temperatures. Several previous studies have reported interaction between spoilage microflora of seafood. For example, it has been documented that siderophore-producing *Pseudomonas* spp. inhibit the growth of *Shewanella putrefasciens*, lowering its maximum concentration in fish stored at 0ºC up to 2 log CFU/g (Gram, 1993; Gram et al., 2002; Gram and Melchiorsen, 1996). The cooking effect on physico-chemical characteristics of shrimps rendering some nutrients (e.g. amino acids) more easily accessible to the microorganisms may explain why the growth rate of *Pseudomonas* spp. was lower in fresh shrimps in comparison to *P. psychrophila* in cooked shrimps. Another reason could be the

interaction among endogenous flora in fresh shrimps and the reduction in maximum concentration of microorganisms by overgrowing flora which is known as the Jameson effect (Gram et al., 2002; Jameson, 1962; Mellefont et al., 2008). In a previous study, we found that at high storage temperatures, H_2S-producing bacteria were the dominant group of microorganisms associated with shrimps caught from the same lake (Dabadé et al., 2015). We found similar growth rates of *Pseudomonas* spp. in fresh shrimps inoculated with *P. psychrophila* and fresh non-inoculated shrimps (results not shown). This similarity could be explained by the fact that the inoculated *P. psychrophila*, found to be the fastest growing *Pseudomonas* spp. spoiling fresh shrimps (Table S5.1), was previously isolated from the fresh sample and it might be likely to be associated with non-inoculated shrimps in this study. It is known that in a mixed population, the growth rate of the population is similar to that of the fastest growing organism (McMeekin et al., 1993).

The initial total volatile basic nitrogen value (TVBN) found in fresh shrimp (*Penaus notialis*) in this study (29.2 mg/100 g) was similar to the one found in a previous study (Dabadé et al., 2015) on the same species of shrimp collected in the same lake (30.1 mg/100 g) and to the value (30 mg/100 g) found in pink shrimp (*Parapenaeus longirostris*) caught in Spain (López-Caballero et al., 2007). The initial value of TVBN in cooked shrimp was significantly lower than in fresh shrimp probably because some nitrogen compounds evaporated or were released into the cooking water as previously demonstrated (Cambero et al., 1998). The TVBN values at the sensory rejection times (43-54 mg/100 g) of cooked shrimps were significantly lower than those of fresh shrimps (73-85 mg/100 g). This suggests that TVBN standard rejection values of a given seafood product should take into consideration the processing undergone by the product. It would also indicate that compounds in TVBN may differ for cooked or fresh shrimps or that other compounds are involved in rejection.

The conceptual minimal temperature T_{min} for pseudomonad growth found in this study (Table 5.1) is lower than those previously reported (-8 to -5ºC) for the same group of microorganisms (Gospavic et al., 2008; Neumeyer et al., 1997). However, similar value of T_{min} for pseudomonad growth in fish (*Sparus aurata*) has been reported by Koutsoumanis (2001). According to this author, strains variability and differences in the structure and the composition of the growth medium (real food versus laboratory medium) are some reasons that could explain the low value of T_{min} found.

According to Dalgaard (2000), a B_f in the range of 0.75-1.25 indicates a successful evaluation of seafood spoilage models. The validation of the model (Eq. (5.6)) predicting fresh shrimp shelf-life at constant storage temperatures (Table 5.2) shows that the model can be used to successfully predict the shelf-life of fresh shrimps as well as cooked shrimps inoculated with *P. psychrophila*. However, with a B_f value of 0.61, the shelf-life of cooked shrimps inoculated with *C. maltaromaticum* cannot be predicted well by the model. The acceptable prediction zone method (Fig. S5.3) also confirmed this since all the relative errors for the shelf-life of cooked shrimps inoculated with *C. maltaromaticum* were outside the acceptable prediction zone. Although the model can be used to support shrimp stakeholders' decisions regarding shrimp quality management, it cannot predict shelf-life under fluctuating temperature, which often occurs in the supply chain. Moreover, the model cannot take into account the effect of the initial microbial concentration on shrimp shelf-life. Fortunately, these shortcomings are taken into consideration in the models predicting the growth of pseudomonads in shrimp under non-isothermal conditions. To construct these models, the uncertainty of the predictions due to the lag phase was simulated with extremes of the initial physiological state of the microorganisms. Similar approach was also used by Bovill et al. (2001). For steps in the exponential phase, often no (large) lag phases are observed as a result of temperature shift (Bovill et al., 2001; Zwietering et al., 1994b), and also in this study, no clear deviations were observed after growth was initiated. The validation of these models show that they can be used to successfully predict shrimp shelf-life under fluctuating temperature since the B_f values obtained (Table 5.3) ranged between 0.75-1.25 (Dalgaard, 2000). The good agreement found between the predicted and observed shelf-life of shrimp during both temperature profiles (maximum 15% difference between the predicted and the observed shelf-life, Table 5.4) confirmed that the shelf-life of shrimp can be estimated from pseudomonads growth data at different storage temperatures, especially at low temperatures where they are dominant microorganisms. Although the

data at 28°C could not be fitted since the growth was so fast that not enough data points were available in the exponential phase, the data were used for the validation of the prediction of *Pseudomonas* counts at 28°C (data not shown). The prediction of the counts was based on the maximum specific growth rate estimated at 28°C by extrapolation in fresh non-inoculated shrimps (Fig. 5.4B). With a B_f of 1.07 and an A_f of 1.30, the validation showed that the prediction was successful (Dalgaard, 2000). Although shrimp shelf-life prediction was based on *Pseudomonas* counts, TVBN production could also serve as basis for shrimp shelf-life prediction given the high correlation between both parameters. The relationship between *P. psychrophila* concentration and the production of TVBN is shown in Fig. S5.4.

The observed shelf-life of fresh shrimp stored under temperature profile 1 (28/10/5ºC) where temperature abuse occurred in the first part of the chain (28°C, 6 h), was only 30 h even though after these first 6 hours at 28°C, shrimps were stored at lower temperatures, namely 10ºC (for 12 h) and 5ºC (for 12 h). This shelf-life was even shorter than the shelf-life of shrimps stored at a higher constant temperature (15ºC) (43 h) although they contained similar initial microbial concentrations. A similar observation was reported by Bruckner et al. (2012), who found a remarkable shelf-life reduction of fresh pork and poultry due to temperature abuse in the beginning of storage. Our study highlighted the necessity of cooling shrimps as soon as they are caught. Maintaining the cold chain is also of importance. For example, the observed shelf-life of fresh shrimps stored at a constant temperature of 0ºC was 11 d. Alternating shrimp storage temperature between 0ºC (for 24 h) and 7ºC (for 24 h) (temperature profile 2) reduced the observed shelf-life by almost 50% (6 d).

In conclusion, combining microbiological, chemical and sensory analyses, this study demonstrated that overall, the spoilage activity of *P. psychrophila* in tropical shrimp (*Penaeus notialis*) is higher than that of *C. maltaromaticum*. The validation of the model previously developed (Eq. (5.6)) showed that this model can be used as tool to adequately predict the shelf-life of tropical shrimps during storage at constant temperatures. Models predicting the growth of pseudomonads as a function of temperatures were developed. Their validation showed that they are able to predict the shelf-life of shrimps in satisfactory manner under dynamic storage temperatures based on the time required by spoilage organisms to reach the spoilage level.

Acknowledgments

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Supplementary materials

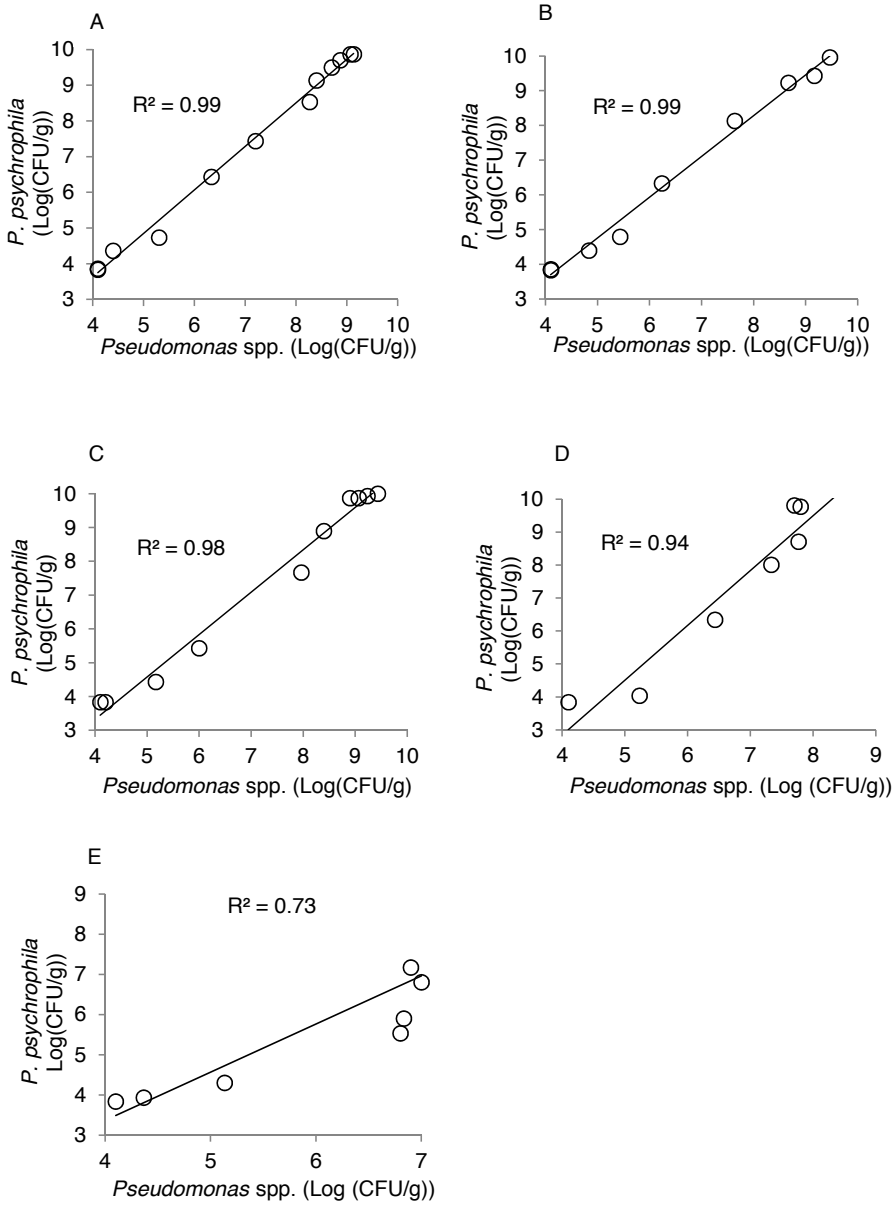

Fig. S5.1 Relationship between the mean concentration of *Pseudomonas psychrophila* in cooked shrimps and the mean concentration of *Pseudomonas* spp. in fresh shrimps inoculated with *Pseudomonas psychrophila* during storage at 0ºC (A), 4ºC (B), 7ºC (C), 15ºC (D), and 28ºC (E).

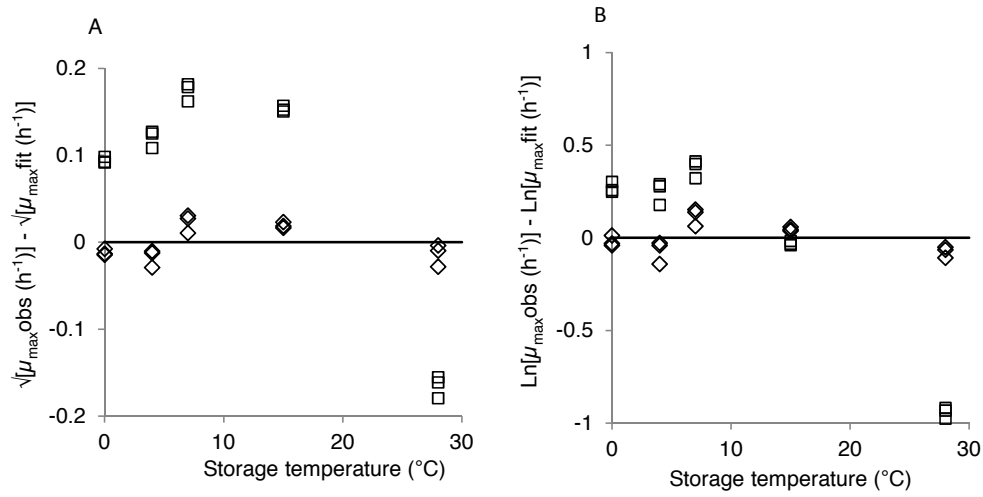

Fig. S5.2 Residuals plot ($\sqrt{\mu_{\max} observed} - \sqrt{\mu_{\max} fitted}$) (A) and ($\ln \mu_{\max} observed - \ln \mu_{\max} predicted$) (B) of *Pseudomonas psychrophila* in cooked shrimps against storage temperatures. The fitting was performed using the Ratkowsky equation (◇) and the Arrhenius equation (□).

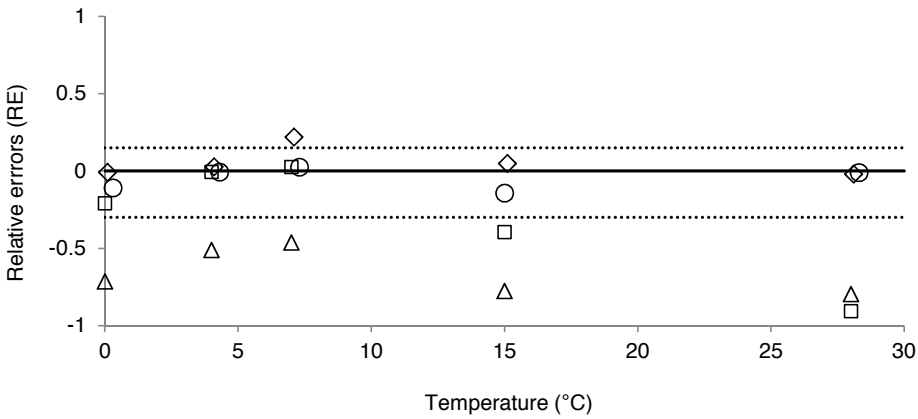

Fig. S5.3 Relative errors for shrimp shelf-life (days) (see Eq. (5.10)) plot against storage temperature with an acceptable prediction zone from an RE of -0.30 to 0.15. The dotted lines indicate the boundaries of acceptable prediction zone.

Legend: (□) = cooked shrimps inoculated with *Pseudomonas psychrophila*, (△) = cooked shrimps inoculated with *Carnobacterium maltaromaticum*, (◇) = fresh shrimps inoculated with *Pseudomonas psychrophila*, (○) = fresh non-inoculated shrimps.

Table S5.1

Estimated maximum specific growth of potential spoilage organisms of tropical brackish water shrimps by Bioscreen automated optical density method.

Strain number	Strain name	μ_{max} (h^{-1})
Strain 5	*Pseudomonas psychrophila*	**0.27**
Strain 4	*Pseudomonas gessardii*	0.18
Strain 13	*Pseudomonas costantinii*	0.14
Strain 16	*Carnobacterium maltaromaticum*	**0.13**
Strain 15	*Acinetobacter johnsonii*	0.11
Strain 18	*Vagococcus fluvialis*	0.05
Strain 20	*Enterococcus faecalis*	0.04

Note

A series of five two fold dilutions were incubated in duplicate per isolate in the wells of a 100-well honeycomb plate containing 200 μl of BHI broth. The initial cell concentration was approximately 10^4 CFU/ml. The honeycomb plates were incubated in the automated Bioscreen C at 7°C for 17 days and the turbidity of the culture was measured at the OD_{600} every 30 min.

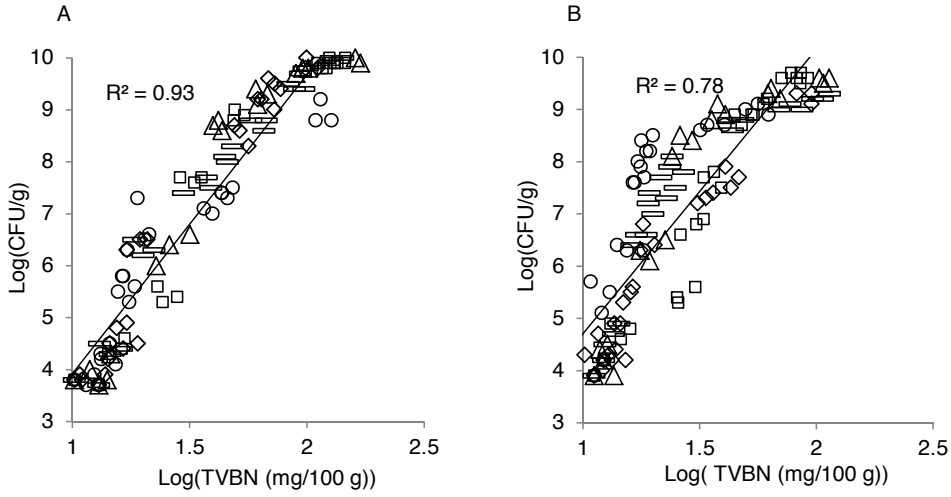

Fig. S5.4 Relationship between the increase in *Pseudomonas psychrophila* number and the production of TVBN in cooked during storage at 0ºC (◇), 4ºC (▭), 7ºC (□), 15ºC (△), and 28ºC (○).

6

Microbiological risks classification and safety management of tropical shrimp (*Penaeus* spp.)

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Submitted for publication

Abstract

Shrimps (wild and farmed) are one of the most important fishery products exported from developing countries to the developed world. However, shrimps may be a source of foodborne pathogens, and shrimp consumption might result in food safety risks. In this study, we identified potential microbiological hazards associated with shrimps caught from brackish waters in a tropical region (West Africa). The risks posed by these hazards to the consumers in the importing countries were classified using different though complementary approaches. Vibrio parahaemolyticus *and* Salmonella *were the most important pathogens associated with tropical shrimp consumption. However, the risks introduced by viruses and pathogenic* Escherichia coli *were also high. Quantitative microbiology was used to predict the growth of* V. parahaemolyticus *and* Salmonella *under dynamic storage temperatures simulating realistic temperature fluctuation in the shrimp supply chain as well as their inactivation under various temperatures. The management of the risks posed by the two main pathogens was addressed using different scenarios to meet the set food safety objectives. The study highlights the importance of cooling shrimps as soon as they are being caught and ensuring hygienic practices in the chain from fishing areas to consumption, to control shrimp safety.*

Introduction

Although rich in protein, shrimps are very low in fat and calories, making them a very healthy choice of food (Bono et al., 2012). They are an important commodity in the international fisheries trade and there is an indication of an increase in worldwide consumption of this crustacean (Norhana et al., 2010). About 75% of shrimp production, whether cultured or wild-caught, originates from developing countries, while 70 to 75% of global shrimp consumption occurs in developed countries (Flick and Granata, 2010).

Regulations, standards and procedures imposed by importing countries remain a big challenge to developing countries which export shrimps. Because of the importance of foreign currency to their economy and the number of people employed along the supply chain, exporting developing countries should develop and promote shrimp quality and safety improvement (Dabadé et al., 2014), and microbiological shrimp safety management should be a concern. Indeed, like any other seafood, shrimps can be associated with potential risks, particularly those related to microbiological contamination (Amagliani et al., 2012). In fact, seafood may be a vehicle of most of the known bacterial pathogens (Huss, 1997) and it is high on the list of foods transmitting diseases (Huss et al., 2000). For instance, in 2003 and 2004, more than 1230 cases of gastroenteritis due to the consumption of raw or undercooked shrimps have been reported in Mexico, and the infectious agent was *Vibrio parahaemolyticus* (Cabanillas-Beltran et al., 2006). A salmonellosis outbreak on a commercial cruise ship in 2000 was due to the consumption of shrimp from a South-East Asian country (Koonse et al., 2005).

The objectives of the present study were (i) to identify the pathogens that may be associated with tropical brackish water shrimps; (ii) to classify the risks posed by these pathogens, and (iii) to assess the dynamics of the risk and to suggest different scenarios for shrimp safety management. The focus will be on shrimps caught from brackish waters (lakes and lagoons) in Benin (West Africa), as an example developing country that exports shrimps to developed countries.

Materials and methods

Hazard identification

To identify the potential microbiological hazards associated with brackish water tropical shrimp, we made use of literature and expert knowledge as previously described by Van Gerwen et al. (1997). Briefly, different levels of details were used to identify hazards stepwise, starting with the most relevant hazards.

Risk classification

To classify the risks posed by the identified hazards three different approaches were used namely, the Risk Ranger software (Ross and Sumner, 2002), the rapid alert system for food and feed notifications (https://webgate.ec.europa.eu/rasff-window/portal/), and a mathematical equation determining the number of cases of illnesses based on the exposure to the hazards as described by Zwietering (2005).

Risk classification using Risk Ranger software

Literature data, expert knowledge and our data on the shrimp supply chain enabled us to provide answers to 11 questions from Risk Ranger software for each of the identified hazards. The questions addressed by the software are related to the severity of the hazard, the likelihood of a disease-causing dose of the hazard to be present in the meal, and the probability of exposure to the hazard in a defined period of time. **Questions 1** and **2** are related to the severity of the illness and the susceptibility of the population of interest. The severity of the illness depends on the causative agent and the considered population. For each hazard, we selected one of the choices offered by the software based on literature and expert knowledge. *Listeria monocytogenes* and *Vibrio vulnificus* were not considered to cause illness within all members of the population since the susceptible population for infection caused by *Listeria monocytogenes* is pregnant women, old and young people as well as immuno-compromised people and, for *Vibrio vulnificus*, persons with high levels of serum iron and with liver disorder associated with

high alcohol consumption (ICMSF, 2006). **Questions 3** and **4** of the software deal with the frequency of consumption and the proportion of the population consuming the product. Beninese shrimps are mainly exported to Spain, Belgium and France. We considered the total population of these three countries, which is approximately 122 million (http://worldpopulationreview.com/countries/) as the size of the consuming population. For this risk assessment, it was assumed that the mean serving size is 200 g/meal. The mean serving size of 275 g/meal was used in a previous shrimp microbiological risk assessment (FAO/WHO, 2005) based on an average serving of shrimp consisting of 10 individual shrimps, described as weighing between 25 and 30 g (average 27.5 g) (Winkel, 1998). However, the authors reported that the edible portion will be less than 275 g after the carapace and cephalothorax have been removed prior to consumption. With an exported quantity of about 700,000 kg/annum in Benin the total estimated number of servings per annum in the importing countries by dividing the quantity exported by the mean serving size is 3,500,000. As it was assumed in a previous shrimp microbiological risk assessment (FAO/WHO, 2005), we supposed that of total servings, 10% were consumed raw and 90% consumed cooked. Therefore, of 3,500,000 servings per annum, 350,000 are consumed raw and 3,150,000, cooked. Taking into account the population size (122 million) (**question 5**), it can be estimated that about 0.0029 serving of Beninese shrimp is consumed raw per person per year and about 0.026 serving of Beninese shrimp is consumed cooked in the importing countries. For the shrimp eaten cooked, as suggested by FAO/WHO (2005), rather than saying 100% of the population eat 0.026 serving per year, we could say that 1% of the population eat 2.6 servings of Beninese cooked shrimp per year. By analogy, it can be estimated that 0.1% of the population eat 2.9 servings of Beninese raw shrimp per year. In the Risk Ranger software, we selected for cooked or raw shrimps the lowest available value of the proportion of the population consuming the product, which is 5%. The 2.9 servings per year were expressed as "a few times per year". **Question 6** of the software is related to the probability that a serving of raw product is contaminated. Based on literature and expert knowledge, a probability of contamination of the product per serving varying between 0.005% and 15% was assumed for each group of hazards. It is known that the prevalence of psychrotrophic pathogens such as *Listeria monocytogenes* and non-proteolytic *Clostridium botulinum* in warm water fish is extremely low (Dodds, 1993; Fuchs and Surendran, 1989; Huss, 1997). However, *L. monocytogenes* is known to proliferate and persist in food processing environment (Schonberg and Gerigk, 1991). Therefore, raw shrimp may get contaminated in shrimp processing plants. **Question 7** of the Risk Ranger software asks how the process can affect the hazards. At processing plants in Benin, raw shrimps are washed and frozen (there is no cooking step) (Dabadé et al., 2014). Washing and freezing will reduce microbial concentration in shrimp. Given the fact that the freezing time for a batch of shrimp may last only 24 h in shrimp plants and based on the fact that vegetative cells (e.g. *Salmonella*) can survive a frozen state for a long time (Amagliani et al., 2012; Archer, 2004), we selected the option: "the process SLIGHTLY (50% of case) REDUCES hazards" for spore forming bacteria (*Bacillus cereus, Clostridium botulinum*) and enteric viruses and "the process ELIMINATES 90% of case" for vegetative cells. Freezing is effective for parasite inactivation (Adams et al., 1997; Butt et al., 2004a; Lee and Lee, 2001). Therefore, we selected the option "the process RELIABLY ELIMINATES hazards" for parasites. **Question 8** deals with recontamination after processing. According to FAO/WHO (2005) and Sumner (2011), the retail market in shrimp importing countries provides little opportunity for contamination since the product is stored under frozen conditions. Like these authors, we considered that there is no contamination after processing for the present risk assessment. **Question 9** addresses the effectiveness of post-processing controls. The cold chain (frozen and chilled) is well established in international trade (FAO/WHO, 2005). Therefore, the option "WELL CONTROLLED (no increase in population)" was selected for bacteria. Question 9 is not relevant for viruses and parasites since they cannot grow in food. **Question 10** is about the increase in the post-processing contamination level that would cause infection or intoxication to the average consumer. The question is relevant for bacteria only. As in question 6, based on literature and expert knowledge, an increase in number varying between 10-fold and 10,000-fold was assigned to the identified hazards. **Question 11** is about the effect of meal preparation. For shrimp eaten raw, we selected the option: "meal preparation has NO EFFECT on the hazards".

Risk classification using the rapid alert system for food and feed (RASFF) notifications

The number of notifications namely alert, information, and border rejection notifications by RASFF related to imported tropical shrimp to EU was determined considering a period of 10 years (January 1st 2004 - December 31st 2013).

Risk classification using a mathematical equation

A mathematical equation (6.1) determining the number of cases of illnesses based on the exposure to the hazards as described by Zwietering (2005) was used.

$$N = \left[1 - \exp\left(-C \times M \times r\right)\right] \times P \times n \quad (6.1)$$

N is the number of cases of illnesses, M is the mass of serving (g), C is the concentration of the pathogen (CFU/g), P is the prevalence, n is the number of servings per year in the considered population, r is the estimated probability of a certain effect (illness) when the ingested dose is represented by one cell.

If $C \times M \times r < 0.1$, $\left[1 - \exp\left(-C \times M \times r\right)\right] \approx C \times M \times r$ and Eq. (6.1) becomes

$$N = M \times C \times P \times n \times r. \quad (6.2)$$

The dose-response parameter r is not yet determined for some pathogens. Only pathogens with a known r value were therefore taken into account in this risk assessment. It is likely that the concentration of the microorganisms in shrimp after the processing under the conditions stated with the Risk Ranger software (no recontamination after processing, post-processing control system well controlled) is low. We assumed that this concentration is 10 CFU/g for *Salmonella*, pathogenic *E. coli*, *V. cholera,* and *L. monocytogenes*, 100 CFU/g for *V. parahaemolyticus* (since the prevalence of this organism is higher than that of other *Vibrio* species in the coast of West Africa (Bockemuhl and Triemer, 1974; Chigbu and Iroegbu, 2000) and 10 particles of viruses per gram of shrimps. We used the same prevalence values as used with the Risk Ranger software.

Quantitative microbiology

Prediction of growth rate as function of temperature

Growth rate dependency on temperature was assumed to follow the Ratkowsky equation (Eq. (6.3)) (Ratkowsky et al., 1982).

$$\sqrt{\mu_{\max}} = b\left(T - T_{\min}\right) \quad (6.3)$$

where $\mu_{\max}$ is the maximum specific growth rate (per hour), T is the temperature (in degrees Celsius), and b and $T_{\min}$ are the regression coefficients to be estimated. $T_{\min}$ (in degrees Celsius) is the extrapolated lowest temperature at which a microorganism cannot grow anymore. Two approaches were used to model the growth rate as function of temperature.

Gamma-concept

With the Gamma-concept, the b value of the Ratkowsky equation (Eq. (6.3)) can be estimated (Eq. (6.4)) (Zwietering et al., 1996).

$$b = \sqrt{\gamma(pH) \cdot \gamma(a_w) \frac{\mu_{opt}}{\left(T_{opt} - T_{min}\right)^2}} \quad (6.4)$$

with

$$\gamma(pH) = \frac{\left(pH - pH_{min}\right)\left(2 \cdot pH_{opt} - pH_{min} - pH\right)}{\left(pH_{opt} - pH_{min}\right)^2} \quad (6.5)$$

$$\gamma(a_w) = \frac{a_w - a_{w,min}}{1 - a_{w,min}} \quad (6.6)$$

The average pH of fresh shrimp is 7.4 (Dabadé et al., 2015a) and the water activity is estimated to be 0.97.

For the calculation, we used T_{min}5°C, T_{opt}36.8°C, pH_{min}4.5, pH_{opt}7.9, $a_{w,min}$0.94, μ_{opt}4.62 h^{-1} for *V. parahaemolyticus* and, T_{min}5°C, T_{opt}37.7°C, pH_{min}3.7, pH_{opt}7.1, $a_{w,min}$0.93, μ_{opt}2.31 h^{-1} for *Salmonella* (ICMSF, 1996).

ComBase program

The *Salmonella* growth model available in the ComBase program (www.combase.cc) was used (ComBase prediction is not yet available for *V. parahaemolyticus*). In the program, the *pH* was set at 7.4 as with the Gamma-concept, and the a_w at 0.975, the minimum value allowed by the program. Then, the maximum growth rates (μ_{log}, $\log_{10}(\text{CFU})/\text{h}$) at six different temperatures ranging between 7 and 40°C generated by the program were collected and multiplied each by ln(10) to obtain the maximum specific growth rates (μ_{max}) at these temperatures. The Ratkowsky equation was fitted to the maximum specific growth rates to estimate the parameters *b* and T_{min}.

The predicted growth rates were compared to data from the literature related to seafood products as well as meat products in case of *Salmonella*, because published kinetic data on the growth of *Salmonella* in seafood are scarce.

Modeling procedure

The Ratkowsky parameters predicted by the Gamma-concept (b = 0.0473, T_{min} = 5°C for *V. parahaemolyticus;b* = 0.0350, T_{min} = 5°C for *Salmonella*) were used to predict bacterial growth under dynamic temperature regimes simulating temperature fluctuation along the supply chain. We assumed that the initial concentrations N_0 of *V. parahaemolyticus* and *Salmonella* spp. in shrimp were 2 log CFU/g and 1 log CFU/g, respectively. The lag-exponential model (Eq. (6.7)) (Van Gerwen and Zwietering, 1998) was used for microbial growth prediction.

$$\log_{10}(N_t) = \log_{10}(N_0) \text{ for } t < \lambda$$

$$\log_{10}(N_t) = \log_{10}(N_0) + \frac{\mu_{max}}{\ln(10)} \cdot (t - \lambda) \text{ for } \lambda < t < t_{max} \quad (6.7)$$

where t is the time (h), N_t is the number of microorganisms at time t (CFU/g), N_0 is the number of microorganisms at time zero (CFU/g), λ is the lag time (h), t_{max} is the time when the maximum number of microorganisms is reached (h), μ_{max} is the maximum specific growth rate (per hour).

To estimate the lag phase, we assumed the product $\mu_{max} \times \lambda$ to be between 1 and 4 (Dabadé et al. 2015b; Koutsoumanis et al., 2006; Zwietering et al., 1994a). It was also assumed that there is no additional lag time when a temperature shift occurred during the exponential phase and a temperature shift within the lag phase can be considered by adding relative parts of the lag time still to be completed (Zwietering et al., 1994b).

Our investigation in the Beninese shrimp supply chain revealed that 94.1% of fishermen were not using ice (Dabadé et al., 2014). Thus, shrimps were kept at ambient temperature (28°C) up to 6 h before their storage in ice using non-isothermal containers. Shrimp temperature may reach 10°C at the reception in shrimp plants. However, a new system of shrimp collection and transportation in which isothermal containers are used to maintain shrimp temperature constant at 0°C during transportation is being promoted. In this new system, fishermen are encouraged to store shrimps in ice as soon as they are being caught. The transportation to shrimp processing plants may last 3 h (Dabadé et al., 2014). In shrimp processing plants, shrimp temperatures do not exceed 7°C, and in general, it takes about 4 h for a batch of shrimps received at the plants to undergo the processing steps previously described (Dabadé et al., 2014) prior to the freezing step. Based on this information, three scenarios were selected for this study. During the first scenario, shrimps were stored first at 28°C for 6 h, afterward, they were stored at 10°C for 3 h and at 7°C for 4 h (scenario 28/10/7). During the second scenario, shrimps were stored first at 28°C for 6 h, afterward, they were maintained at 0°C for 3 h and at 7°C for 4 h. (scenario 28/0/7). Finally, in the third scenario, shrimps were maintained at 0°C for 9 h (shrimp fishing, collection and transport time) then at 7°C for 4 h (scenario 0/7).

Inactivation rate estimation

The Bigelow model (Eq. (6.8)) (Bigelow, 1921) was used to describe the effect of temperature on the decimal reduction time of the microorganisms.

$$\log D = \log D_{\text{ref}} - (T - T_{\text{ref}})/z \tag{6.8}$$

D is the heating time needed to obtain a 1-log reduction (min), log$D$ is the logarithm of the D-value (log min), logD_{ref} is the log$D$-value at T_{ref} (log min), T_{ref} is the reference temperature (°C), and z is the temperature increase (°C) needed to reduce the D-value by a factor of 10. We used for *V. parahaemolyticus* $\log(D_{70}) = -1.30$, $z = 8.5$, and for *Salmonella* spp. $\log(D_{70}) = 0.59$, $z = 9.1$°C (Van Asselt and Zwietering, 2006). These are the 95 upper percentiles of the $\log(D_{70})$ values to be on the safe side.

Shrimp safety management: determination of food safety objectives (FSOs)

The FSO concept can be used to make food safety transparent and quantifiable (Zwietering, 2005). The concept relies on an appropriate level of protection (ALOP).

As described in Eq. (6.2), the number of illnesses N per year per million people can be estimated using Eq. (6.9)

$$N = M \times C \times P \times S \times r \times 1{,}000{,}000 \tag{6.9}$$

with S is the number of serving per person per year.

ALOP = deaths per million people per year

ALOP = number of illnesses per million per year (N) × mortality rate (q)

$$C \times P = \frac{\text{ALOP}}{M \times S \times r \times q \times 1{,}000{,}000} \tag{6.10}$$

$$\text{FSO} = \log(\text{C} \times \text{P}) = \log(C) + \log(P)$$

If all the units of the product are contaminated ($P = 1$), then $\text{FSO} = \log(C)$.

Let us assume that the ALOP is 0.05 death per million people per year, for shrimps. This assumption is based on the estimated current level of protection for *Salmonella* in fish and shellfish, which is 0.8 death per year in a population of 17 million (Bouwknegt et al., 2012). For the calculation, we used the same values of M and r as with Eq. (6.1), a q value of 0.025 and 0.0078 were used for *V. parahaemolyticus* and *Salmonella*, respectively (Mead et al., 1999). The number of servings per person per year varies from one country to another within the E.U. Spain is known as the major market for shrimps within the E.U. The number of serving per person per year (S) in that country was used in this study. To estimate S, the quantity of shrimps consumed on average annually in Spain calculated from data published by CBI (2010) was divided by the mass of serving M to obtain the total number of servings per year in the country. This value was then divided by the population size to obtain the average number of servings per person per year S which was 15.5.

In a food supply chain, the FSO can be derived from Eq. (6.11) (ICMSF, 2006).

$$H_0 - \sum R + \sum I \leq \text{FSO} \tag{6.11}$$

where, H_0 is the logarithm of the initial concentration (log N_0/g), $\sum R$ is the total (cumulative) log reduction (per g) of the pathogen, $\sum I$ is the total (cumulative) log increase (per g) of the pathogen due to growth or recontamination, FSO is the Food Safety Objective (log CFU/g). At each step of a food supply chain, the FSO approach can be applied and the maximum tolerable concentration at any step prior to the point of the consumption is called Performance Objective (PO). In this study, the results of quantitative microbiology were used in different scenarios along the supply chain for FSO achievement.

Results

Potential biological hazards associated with tropical brackish water shrimps

Combining literature and expert knowledge the potential hazards associated with tropical brackish water shrimps and their origins are presented in Table 6.1.

Table 6.1

Potential hazards associated with tropical brackish water shrimps and their origins

Groups of hazards	Hazards	Origins[a]
Bacteria	*Aeromonas*	1
	Bacillus cereus	1, 3
	Clostridium botulinum	1
	Escherichia coli	2
	Listeria monocytogenes	1, 3
	Salmonella	2
	Staphylococcus aureus	2, 3
	Vibrio cholerae	1
	Vibrio parahaemolyticus	1
	Vibrio vulnificus	1
Viruses	Hepatitis A	2
	Norovirus	2
Parasites	Helminths	2
	Protozoa	2

[a]1 = naturally present in aquatic environment, 2 = animal and human origin, 3 = handling or processing.

Table 6.2
Risk associated with consumption of raw shrimp contaminated with (A) bacteria and (B) viruses and parasites estimated by Risk Ranger

A

Risk Ranger parameter	*Aeromonas*	*B. cereus*	*C. botulinum*	*E. coli*	*L. monocytogenes*	*Salmonella*	*S. aureus*	*Vibrio cholerae*	*V. parahaemolyticus*	*V. vulnificus*
Q1	Mild	Mild	Severe	Moderate	Severe	Moderate	Mild	Moderate	Mild	Moderate
Q2	General	General	General	General	Slight	General	General	General	General	Very
Q3	A few times a year									
Q4	Very few									
Q5	122 million									
Q6	15%	1%	0.01%	0.1%	0.1%	1%	10%	1%	15%	1%
Q7	Eliminates hazards in 90% of cases	Slightly (50% of cases) reduces hazards	Slightly (50% of cases) reduces hazards	Eliminates hazards in 90% of cases	Eliminates hazards in 90% of cases	Eliminates hazards in 90% of cases s	Eliminates hazards in 90% of cases	Eliminates hazards in 90% of cases	Eliminates hazards in 90% of cases	Eliminates hazards in 90% of cases
Q8	No									
Q9	Well controlled									
Q10	10000-fold increase	10000-fold increase	10000-fold increase	10-fold increase	10000-fold increase	100-fold increase	10000-fold increase	1000-fold increase	1000-fold increase	1000-fold increase
Q11	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Predicted illnesses/ year	27.5	9.2	0.09	183	0.2	183	18.3	18.3	275	16.5
Risk ranking (0-100)	30	28	33	41	35	41	29	35	36	35

Q1, hazard severity; Q2, susceptibility of population; Q3, product consumption frequency; Q4, proportion of population consuming the product; Q5, size of consuming population; Q6, probability of contamination of raw product; Q7, effect of processing on the hazard; Q8, potential for recontamination of product after processing; Q9, effectiveness of post processing control system; Q10, increase of the hazard level needed to cause illness; and Q11, preparation of product before eating.

B

Risk Ranger parameters	Viruses (Hepatitis A and Norovirus)	Parasites (Helminths and Protozoa)
Q1	Mild	
Q2	General	
Q3	A few times a year	
Q4	Very few	
Q5	122 million	
Q6	0.005%	1%
Q7	Slightly (50% of cases) reduces hazards	Reliably eliminates hazards
Q8	No	
Q9	Not relevant	
Q10	None	
Q11	No effect	
Predicted illnesses/ year	458	0
Risk ranking (0-100)	37	0

For details about the Risk Ranger parameters, see Table 6.2A.

Shrimp microbial risk assessment

The risk ranking summary for the identified hazards using the Risk Ranger software is shown in Table 6.2. The Risk Ranger software is a tool which can be used to give weight to the different risks in the evaluated product and aids to pre-screen the risks that certain pathogens can represent to a product (Ross and Sumner, 2002). The risk ranking is on a logarithmic scale of 0 to 100. Zero represents no risk and 100 represents every member of the population eating a meal which contains a lethal dose of the hazard every day. An increment of six in the ranking corresponds approximately to a 10-fold increase in risk (Sumner et al., 2005). In the present study, the risk rankings of hazards associated with the consumption of Beninese raw shrimp in the imported countries ranged between 0 (for parasites) and 41 (*Salmonella* or *E. coli*). The highest predicted number of illnesses in the population of interest (458 per year) was obtained with viruses. For bacteria, the most important numbers of illnesses were obtained with *V. parahaemolyticus* (275 per year) and *Salmonella* (183 per year) or pathogenic *E. coli* (183 per year).

Based on RASFF notifications (Fig. 6.1), *Vibrio* species, namely *V. parahaemolyticus* and *V. cholerae*, and *Salmonella* were the most incriminated pathogens in tropical shrimp exported to the EU between January 2004 and December 2013. There was no notification on viruses and *E. coli*.

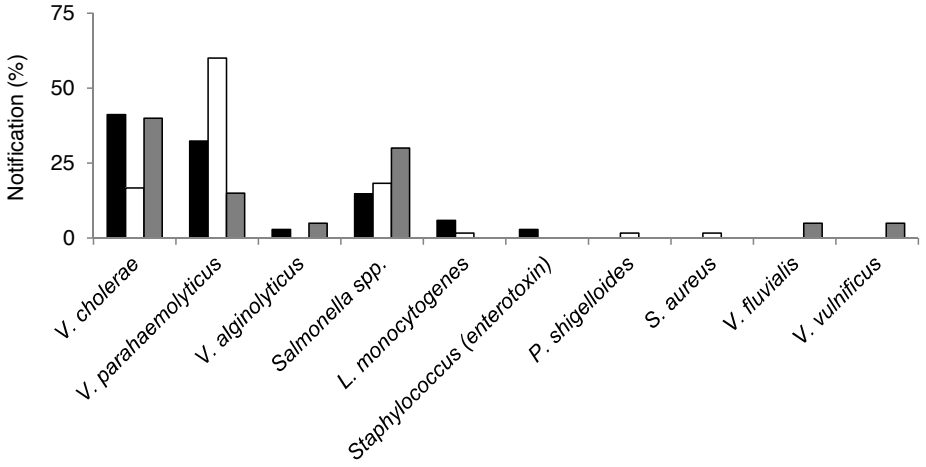

Fig. 6.1 RASFF notifications (%) related to tropical shrimps imported to EU from 01/01/2004 to 31/12/2013. Black bars indicate alert notifications (n=34), grey bars indicate border rejection notifications (n=20), and white bars indicate information notifications (n=60). The x-axis indicates the incriminated pathogens or toxins.

The results of shrimp microbial risk assessment by a mathematical equation (Zwietering, 2005) are presented in Table 6.3. The highest number of predicted illnesses (1,829 per year) was found with *Salmonella*. High numbers of illnesses were also predicted with *V. parahaemolyticus* (481 per year) and *E. coli* (216 per year). Although for most of the pathogens, the number of predicted illnesses by the mathematical equation was higher than that obtained with Risk Ranger software, both approaches gave similar predictions for *E. coli*, *V. parahaemolyticus*, and *V. cholerae* as shown in Fig. 6.2.

Table 6.3

Number of predicted illnesses in the population of interest using Eq. (6.1)

Microorganisms	Mass of serving M (g)	Conc. C[a]	Prevalence P	Number of raw shrimp servings per year n	Dose-response parameter r[b]	Number of predicted illnesses N per year
E. coli	200	10	0.001	350,000	4.8×10^{-4}	216
L. monocytogenes	200	10	0.001	350,000	5.95×10^{-11}	4.2×10^{-5}
Norovirus	200	10	0.00005	350,000	1.7×10^{-1}	17.5
Salmonella	200	10	0.01	350,000	3.7×10^{-4}	1,829
V. cholerae	200	10	0.01	350,000	7.33×10^{-6}	51
V. parahaemolyticus	200	100	0.15	350,000	4.6×10^{-7}	481

[a]Concentration (CFU/g) for bacteria, number of particles per gram of shrimp for viruses.
[b]The dose-response parameter is the average of values extracted from different sources (Rose and Gerba, 1991; Stella et al., 2013; Teunis et al., 1996).

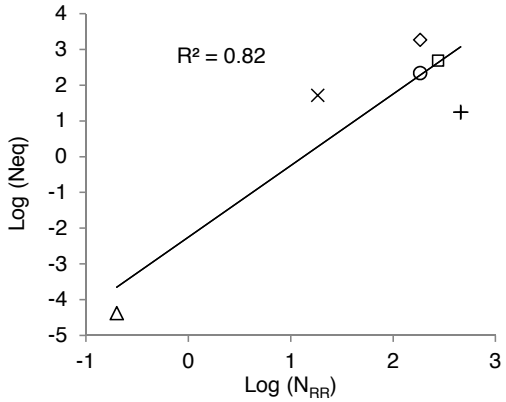

Fig. 6.2 Relationship between the predicted illness per year with Risk Ranger software and a mathematical equation (Zwietering, 2005) by *E. coli* (○), *L. monocytogenes* (△), Norovirus (+), *Salmonella* (◇), *V. cholera* (×), and *V. parahaemolyticus*(□).

Legend: N_{eq}= predicted illness per year by a mathematical equation (Zwietering, 2005)

N_{RR}= predicted illness per year by Risk Ranger software.

Based on the outcomes of the three approaches used for risk assessment, *V. parahaemolyticus* and *Salmonella* can be considered as the main pathogens associated with tropical shrimp consumption.

Quantitative microbiology

Prediction of the growth of Vibrio parahaemolyticus *and* Salmonella *spp. in shrimp*

The parameter *b* (Eq. (6.3)) was estimated to be 0.0473 for *V. parahaemolyticus* and 0.0350 for *Salmonella* using the Gamma concept (Eq. (6.4)). The Ratkowsky model (Eq. (6.3)) was fitted to *Salmonella* growth rates at different temperatures predicted by ComBase and the parameters *b* and T_{min} were estimated to be 0.0346 and 3.8, respectively, so very close to the ones of the Gamma concept (0.0350 and 5). The predicted growth rates using the Gamma concept and ComBase were compared to literature data and results are depicted in Fig. 6.3. In general, there was a good agreement between the predicted models and literature data.

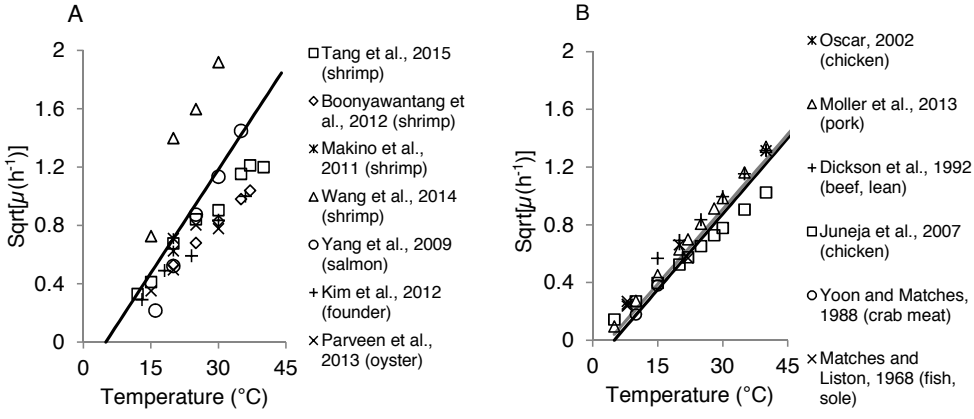

Fig. 6.3 Comparison of the predicted growth rates of *V. parahaemolyticus* (A) and *Salmonella* (B) by the Gamma concept (black solid line) and the ComBase program (grey solid line, only for *Salmonella*) and literature data.

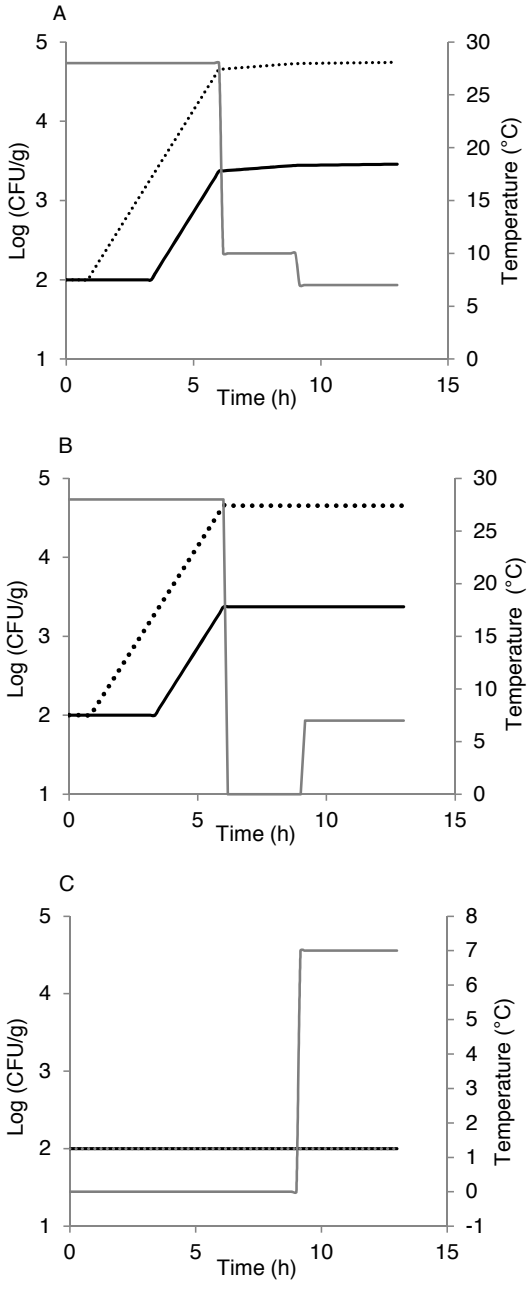

Fig. 6.4 Predicted growth of *V. parahaemolyticus* in fresh shrimp based on the assumption $\mu_{\max} \times \lambda = 1$ (dotted line) and the assumption $\mu_{\max} \times \lambda = 4$ (black solid line) at temperature profile 28/10/7ºC (A), 28/0/7ºC (B), and 0/7ºC (C). Grey solid line indicates temperature profile. The model was constructed using the lag-exponential model (Eq. (6.7)).

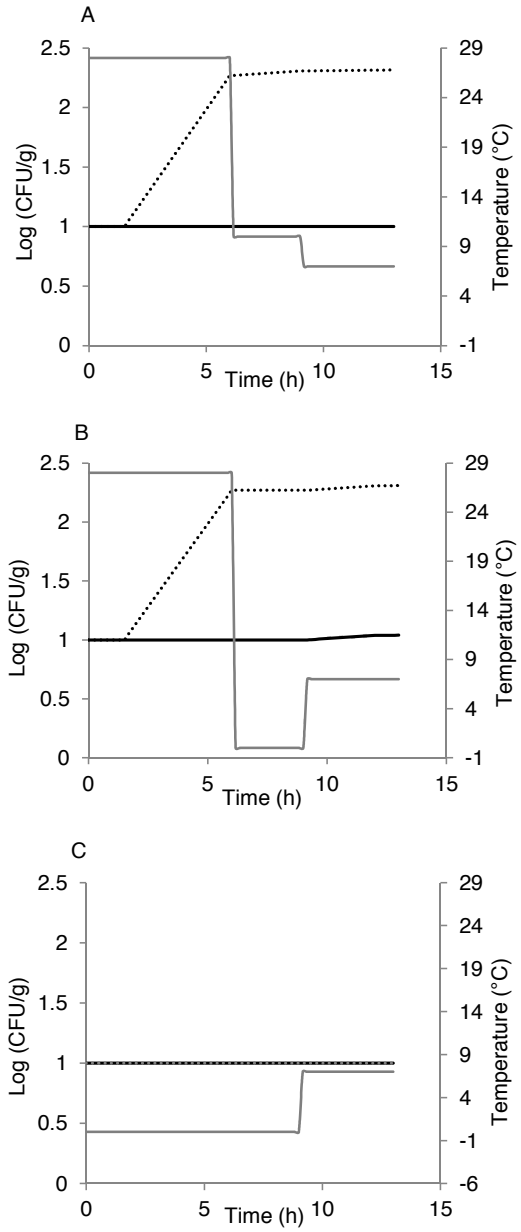

Fig. 6.5 Predicted growth of *Salmonella* in fresh shrimp based on the assumption $\mu_{\max} \times \lambda = 1$ (dotted line) and the assumption $\mu_{\max} \times \lambda = 4$ (black solid line) at temperature profile 28/10/7ºC (A), 28/0/7ºC (B), and 0/7ºC (C). Grey solid line indicates temperature profile. The model was constructed using the lag-exponential model (Eq. (6.7)).

Using the parameters estimated by Gamma-concept (b = 0.0473, T_{min} = 5°C for *V. parahaemolyticus*) (b = 0.0350, T_{min} = 5°C for *Salmonella*), models predicting microbial growth under different temperature regimes simulating temperature fluctuations along the chain were constructed. The predicted number of microorganisms from fishing areas to the step prior to freezing in shrimp processing plants are shown in Fig. 6.4 for *V. parahaemolyticus* and Fig. 6.5 for *Salmonella*. For the scenarios 28/10/7 and 28/0/7, the model predicted an increase in the initial number of *V. parahaemolyticus* (2 log CFU/g) during the first part of the chain where shrimps are stored at ambient temperature (28°C) reaching 3.5 or 4.7 log CFU/g depending on the lag phase. There was no significant increase in the number of *V. parahaemolyticus* during the other steps of the chain prior to the freezing step. With scenario 0/7, no increase in the initial microbial concentration is expected irrespective of the estimated lag time of *V. parahaemolyticus* or *Salmonella* (Fig. 6.4 and 6.5). With the scenarios 28/10/7 and 28/0/7, *Salmonella* (N_0 = 1 log CFU/g) can grow with the shorter lag time estimated reaching 2.3 log CFU/g during the ambient temperature storage. As with *V. parahaemolyticus*, there was no significant growth after the first phase.

Inactivation of Vibrio parahaemolyticus *and* Salmonella *spp. in shrimp*

Using the Bigelow model (Eq. (6.8)), the microbial reduction at various temperatures and exposure times are shown in Table 6.4. *V. parahaemolyticus* is more sensitive to heat treatment than *Salmonella*.

Table 6.4

Log reduction of *V. parahaemolyticus* and *Salmonella* in shrimp exposed to heat treatment at various temperatures for various times

Time (min)	Temperature (°C)	*V. parahaemolyticus*	*Salmonella*
3	50	0.27	0.005
	60	4.0	0.06
	65	15.5	0.22
	75	232	2.7
	80	899	9.7
5	50	0.44	0.008
	60	6.7	0.10
	65	25.8	0.36
	75	387	4.6
	80	1498	16.1
7	50	0.62	0.01
	60	9.3	0.14
	65	36.1	0.51
	75	541	6.4
	80	2097	22.6

Shrimp microbial risk management

The estimated FSO values were found to be -2.85 log CFU/g for *V. parahaemolyticus* and -5.25 log CFU/g for *Salmonella*. To comply with the FSO for *Salmonella* (more stringent than that of *V. parahaemolyticus*), performance objectives and criteria can be set along the supply chain.

Assuming shrimp is eaten raw:

As described in Eq. (6.11), we will have $H_0 - \sum R + \sum I \leq$ -5.25 . Growth prediction model for scenarios 1 or 2 (28/10/7 or 28/0/7) predicted an increase of 1.3 log CFU/g from fishing areas to the step prior to shrimp freezing. The process (washing and freezing) was assumed to produce 1 log reduction as explained in question 7 of the Risk Ranger software. If no recontamination occurs in the supply chain, Eq. (6.11) becomes

$H_0 - 1 + 1.3 \leq -5.25$ or $H_0 \leq -5.55$ log CFU/g. This means that the maximum concentration of *Salmonella* in freshly caught shrimp should be one *Salmonella* cell in approximately 355 kg of shrimp if the prevalence of *Salmonella* in shrimps is 100%. If however, the prevalence is only 1% as assumed for the contamination of raw shrimps (Table 6.2A), then the maximum concentration of *Salmonella* in freshly caught shrimp should be one *Salmonella* cell in approximately 3.6 kg of shrimp.

Assuming shrimp is eaten cooked:

Eq. (6.11) becomes $H_0 + 1.3 - \sum R \leq -5.25$. The reduction due to the process is 1 log CFU/g. So the reduction due to cooking step ($R_{cooking}$) can be calculated as $R_{cooking} \geq H_0 + 5.55$. If the initial concentration of *Salmonella* in shrimp is 1 log CFU/g, then it follows that the cooking step needs to produce a reduction of at least 6.55 log CFU/g (6.55-D reduction). This performance criterion can be achieved by cooking shrimp at 75°C for at least 7 min for example (Table 6.4).

Shrimp is often cooked lightly before consumption (Dalsgaard et al., 1995). If we assume that the cooking step can however result in at least 6-D reduction, different options showing microbial concentration in shrimp along the supply chain are presented in Table 6.5.

Table 6.5

Log reduction of *V. parahaemolyticus* and *Salmonella* in shrimp exposed to heat treatment at various temperatures for various time

Hazards	H_0 (initial concentration, log CFU/g)	$\sum I$	$\sum R$	Total	FSO	Compliance with FSO
V. parahaemolyticus	2	0[a]	7	-5	-2.85	Yes
		2.7[b]	7	-2.3	-2.85	No
	3	0	7	-4	-2.85	Yes
		2.7	7	-1.3	-2.85	No
	4	0	7	-3	-2.85	Yes
		2.7	7	-0.3	-2.85	No
Salmonella	1	0[a]	7	-6	-5.25	Yes
		1.3[b]	7	-4.7	-5.25	No
	2	0	7	-5	-5.25	No
		1.3	7	-3.7	-5.25	No
	3	0	7	-4	-5.25	No
		1.3	7	-2.7	-5.25	No

$\sum I$ is the total (cumulative) log increase (per g) of the pathogen due to growth (no recontamination was assumed)

$\sum R$ is the total (cumulative) log reduction (per g) of the pathogen due to the process (1 log) and the cooking at home (6 log)

[a]predicted growth increase with scenario 0/7 (Fig. 6.5C)

[b]predicted growth increase with scenarios 28/10/7 or 28/0/7 (Fig. 6.5A or 6.5B) and with low lag time (safe side)

FSO is Food Safety Objective (log CFU/g).

This Table shows that with *V. parahaemolyticus*, even an initial concentration of 4 log CFU/g will comply with the FSO set as long as shrimps are cooled immediately after being caught and the temperature is maintained at maximum 7°C along the supply chain. However, if temperature abuse occurs during the first part of the chain (28°C, 6 h) even an initial concentration of only 2 log CFU/g will not comply with the FSO. Immediate cooling is therefore crucial. As for *Salmonella*, the initial concentration should be less than 2 log CFU/g and shrimps should be cooled immediately after being caught to comply with the set FSO.

Discussion

The present study aimed at identifying the main foodborne pathogens associated with tropical shrimps intended for export and to discuss the ways the risks posed by these pathogens can be managed. Hazard identification is the first essential step in Quantitative Microbiological Risk Assessment (QMRA) as well as in Hazards Analysis and Critical Control Points (HACCP), where the specific foodborne pathogens relevant to the considered supply chain are identified (Janevska et al., 2010). Many foodborne pathogens can be associated with shrimp (Reilly and Kaferstein, 1997). According to Van Gerwen et al. (1997), for a given product, it is reasonable to start a risk assessment with pathogens that were reported to have caused foodborne outbreaks in the past. Data on seafood-associated outbreaks are available in the literature (Butt et al., 2004a; Butt et al., 2004b; Iwamoto et al., 2010). Although the mentioned pathogens were not all associated with shrimp specifically, but with seafood in general, they may be considered as potential hazards associated with shrimps.

V. parahaemolyticus which was found to be one of the main foodborne pathogens associated with shrimp in this study is known as a leading cause of seafood-associated bacterial gastroenteritis worldwide (Iwamoto et al., 2010; Quilici et al., 2005; Weissfeld, 2014; Wu et al., 2014).

Salmonella species are also among the most common bacterial cases of food-borne gastroenteritis in humans (Pin et al., 2011). Unlike *Vibrio* species, *Salmonella* species are not believed to be naturally present in the aquatic environment (Dalsgaard et al., 1995). However, they can enter the aquatic environment through wild animals, domestic stock, poor sanitation, and inappropriate disposal of human and animal waste (Amagliani et al., 2012). Salmonellosis outbreaks due to the consumption of seafood occur sporadically (Bean et al., 1997). The brackish waters from where shrimps are caught in Benin are in the vicinity of cities, and people live in pole-houses above some parts of the brackish waters. Thus, contamination of fresh shrimp with *Salmonella* can occur. Although it is not common to detect pathogenic *E. coli* in seafood (Costa, 2013; Kumar et al., 2005; Thampuran et al., 2005), this bacterium was found to be one of the main pathogens in this study by two of the three approaches used (Risk Ranger software, and the mathematical equation). This could be explained by high infectivity of the bacterium so pathogenic *E. coli* growth in food may not be needed to cause illness (Stella et al., 2013). Therefore, when hygienic practices are not followed along the supply chain, this bacterium can be a real threat to people consuming raw or undercooked seafood.

It is not surprising to find viruses as causative agents of high numbers of predicted illnesses per year using Risk Ranger software. In fact, viruses alone are responsible for 50% of the illnesses due to the consumption of seafood particularly, raw and undercooked seafood (Butt et al., 2004b). However, seafood-related infections leading to hospitalizations and deaths are mostly due to bacteria but not to viruses (Butt et al., 2004b). Viruses may therefore be seen as not the most important pathogens and this could justify why they were not reported in RASFF notifications during the considered period. Another reason for not reporting viruses could be their not yet optimal detection methods. For instance, noroviruses cannot be cultured reliably (Lee and Jeong, 2004)

Pathogens associated with a food commodity can increase or decrease in numbers along the supply chain. With predictive microbiology, models describing growth and inactivation of microorganisms can be developed. The Gamma concept used in this study, has been proved to satisfactory predict microorganisms' growth rates as function of various variables (Wang et al., 2012; Wijtzes et al., 2001; Zwietering et al., 1996). In this study, the Gamma concept gave similar growth rate predictions as the ComBase program for *Salmonella*. Previous studies also reported no substantial difference in growth prediction by the Gamma concept and other general models namely pathogen modeling (PMP), Food MicroModel (FMM) (Te Giffel and Zwietering, 1999; Zwietering et al., 1996) and ComBase program (Sosa-Mejia et al., 2011). The overall good agreement obtained between the growth rates from literature and the growth rates predicted by the Gamma concept supports the statement that the Gamma concept can be used to quantitatively describe microorganisms' growth rates in an efficient way (Van Gerwen and Zwietering, 1998). Despite the simplicity of the lag-exponential model used in

this study to describe microbial growth, it has been demonstrated that this model could provide good predictions just as more complicated models such as Gompertz or Baranyi models (Van Gerwen and Zwietering, 1998). Using *Bacillus cereus* growth in cooked potatoes, the same authors came to the conclusion that the impact of process variations (for example small temperature variation) was much more important than that of differences between models. Temperature and time scenarios used in this study are those recorded in the actual shrimp supply chain. However, the scenario that suggests an immediate cooling of shrimps after being caught is not yet a common practice in Benin. The present study shows that no increase in the initial concentration of the main pathogens is expected if shrimps are immediately cooled and the temperature maintained at maximum 7°C latter on in the chain (scenario 0/7). It also highlights the importance of immediate cooling to meet the suggested FSO. Fishermen should therefore be encouraged to go for fishing with ice.

The FSO was found to be more stringent for *Salmonella* than *V. parahaemolyticus*. Also, *Salmonella* was found to be more heat resistant. However the growth rate of *V. parahaemolyticus* was higher than that of *Salmonella*. This implies that if shrimps are contaminated by both pathogens, foodborne pathogens risk from *Salmonella* would be higher if the cold chain is maintained along the supply chain to prevent growth. When temperature abuse occurs, the risk from *V. parahaemolyticus* may be higher especially, if shrimps are eaten raw or undercooked.

In this study, for an initial assessment, it was chosen not to apply a stochastic approach given the uncertainty of all potential sources of variability involved (microorganisms and process parameters). Alternatively various scenarios were developed and the results should be considered as order of magnitude estimations.

The study presents the basis for shrimp microbial safety management. From the Risk Ranger software, if the cooking step before consumption can reliably eliminate the hazard, this leads to zero risk (risk ranking = 0) (data not shown). Thus, for *V. parahaemolyticus* and *Salmonella*, microbial shrimp safety should not be an issue if shrimps will undergo an effective cooking step before consumption. If for example, each part of shrimps being cooked can reach 80°C for 5 min for *Salmonella* (more resistant to heat than *V. parahaemolyticus*), this will lead to 16-D reduction (Table 6.4). And, even if just before the cooking step *Salmonella* concentration were 10 log CFU/g (which is non-realistically high), *Salmonella* concentration in the cooked shrimps would be -6 log CFU/g, which is lower than the FSO set (-5.25 log CFU/g). From all hazards identified (Table 6.1) only *Bacillus cereus* and *Staphylococcus aureus* which can produce heat stable toxins may pose safety problems in effectively cooked shrimps.

Shrimps are however, often eaten undercooked or even raw (Dalsgaard et al., 1995) and as such, they represent a potential health risk for consumers. The FSO approach can be used to assign responsibilities throughout the food chain (Zwietering, 2005).

We found that the initial concentration of *Salmonella* in freshly caught shrimps that are intended to be eaten raw should not exceed one *Salmonella* cell in 3.6 kg (assuming 1% prevalence). This might be achieved by preventing the pollution of fishing areas by humans and following hygiene rules during fishing. To prevent microbial growth, freshly caught shrimps should be iced as soon as possible. The common practice in Benin is that fishermen and some intermediate traders keep shrimps at ambient temperature until their delivery to shrimp collectors (Dabadé et al., 2014). This practice should be changed since during this temperature abuse, pathogenic bacteria can grow and render the product unsafe. In this risk assessment, it was assumed that the cold chain is maintained in shrimp processing plants and that no recontamination occurs after processing. This implies that a well-designed pre-requisite program (Good Hygienic Practice, traceability) and a HACCP plan are implemented and well applied. Finally, consumers should be aware that an effective shrimp cooking at home is crucial, especially for shrimps that do not undergo any heat treatment along the supply chain.

In conclusion, using different approaches to assess microbiological risks, this study shows that *V. parahaemolyticus* and *Salmonella* are the main foodborne pathogens of shrimps caught from brackish waters in Benin. However, attention should also be paid to viruses (Norovirus and hepatitis A) and pathogenic *E. coli*.

Quantitative microbiology was applied to predict the increase in numbers concentrations of *V. parahaemolyticus* and *Salmonella* under dynamic temperature regimes simulating temperature fluctuation along the supply chain as well as their inactivation. This quantitative approach allowed to propose different scenarios for shrimp safety management and showed the efficacy of different scenarios in controlling food safety.

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7

Summarizing discussion

Introduction

Food quality is an important issue, yet its definition is not that simple. According to Zwietering et al. (1992), food quality can be defined as the sum of characteristics of a food, namely its organoleptic properties, nutritional value, and safety conditions that satisfy the expectations of consumers and comply with legal standards. Thus, food spoilage and food safety constitute important food quality attributes. Food spoilage may derive from chemical changes that occur in foods and the presence of toxic pollution compounds (e.g. heavy metals, pesticides) may render foods unsafe. However, in fishery products, microorganisms are the major cause of spoilage (Gram and Dalgaard, 2002) and pathogens may be present in fishery products (Huss et al., 2000). The impact of microorganisms on the quality of foods depends on the food characteristics, and their ability to survive and multiply under the conditions that prevail along the supply chain. Anthropic actions along the supply chain can make the conditions favorable for proliferation of microorganisms. The microbiota of fishery products may also be affected by the ecology of their aquatic environment. For microbial food quality control, insight is needed in the factors that determine the types and the levels of microorganisms that can be associated with the food under the storage conditions or at the time of consumption. Quantitative microbiology can be applied to understand and predict the response of microorganisms to factors affecting the growth and survival in foods.

In this thesis, the quality of tropical brackish water shrimp was investigated along the supply chain. Tools and strategies to improve shrimp quality management were developed based on quantitative and ecological studies. In this chapter, the main findings of the thesis are integrated, discussed in depth and placed in perspective.

Fishery products quality management

Due to their high water activity, neutral pH, relatively high content of free amino acids, and presence of autolytic enzymes, fresh fishery products are highly prone to deterioration (Duan et al., 2010; Jeyasekaran et al., 2006; Zeng et al., 2005). Microorganisms are the major cause of this deterioration and it is well established that only a fraction of microorganisms associated with fishery products really contribute to spoilage (Dalgaard, 1995; Gram and Dalgaard, 2002; Gram and Huss, 1996). This fraction of the spoilage microbiota is termed specific spoilage organism(s) (SSOs) of the fishery product (Dalgaard, 1995). Knowledge of SSOs and their metabolites that can be used as chemical indicators of spoilage is of importance to develop strategies and tools for better fishery products quality management. In chapters 4 and 5 of this thesis, we combined microbiological, chemical and sensory analyses to determine SSOs of tropical brackish water shrimp (*Penaeus notialis*). At low storage temperatures (0-4°C), *Pseudomonas* spp. especially *Pseudomonas psychrophila*, the fastest grown species, was found to be the SSO of *P. notialis*. At high storage temperatures (7-28°C), H_2S-producing bacteria, represented mainly by lactic acid bacteria, and *Enterobacteriaceae* were the main *P. notialis* spoilage organisms. These results suggest that when the cold chain is maintained along the supply chain, *Pseudomonas* spp. are the main spoilage organisms to deal with. However, when temperature abuse occurs, strategies to control shrimp spoilage should target H_2S-producing bacteria and *Enterobacteriaceae*. The H_2S-producing bacteria were composed mainly of lactic acid bacteria instead of *Shewanella* species commonly found to be H_2S-producing bacteria associated with fishery products spoilage. The spoilage lactic acid bacteria were not isolated from de Man, Rogosa and Sharp agar (MRSA) medium widely used to enumerate LAB, but from iron agar. This finding supported the hypothesis that LAB could have been disregarded in fishery products because MRSA is not suitable for the growth of some members of LAB (Leroi, 2010).

When knowledge about the spoilage characteristics of a fishery product is available, tools and strategies can be developed for better quality management. For example, models can be developed to predict the remaining shelf-life at any stage of the food supply chain. In chapter 4, we developed a model that predicts the shelf-life of shrimp at constant storage temperatures (Eq. (7.1)).

$$\text{Shelf-life (days)} = 4.5\times10^{-15}\times\exp\left(\frac{9650}{\theta}\right) \quad (7.1)$$

where θ is the storage temperature in degrees Kelvin.

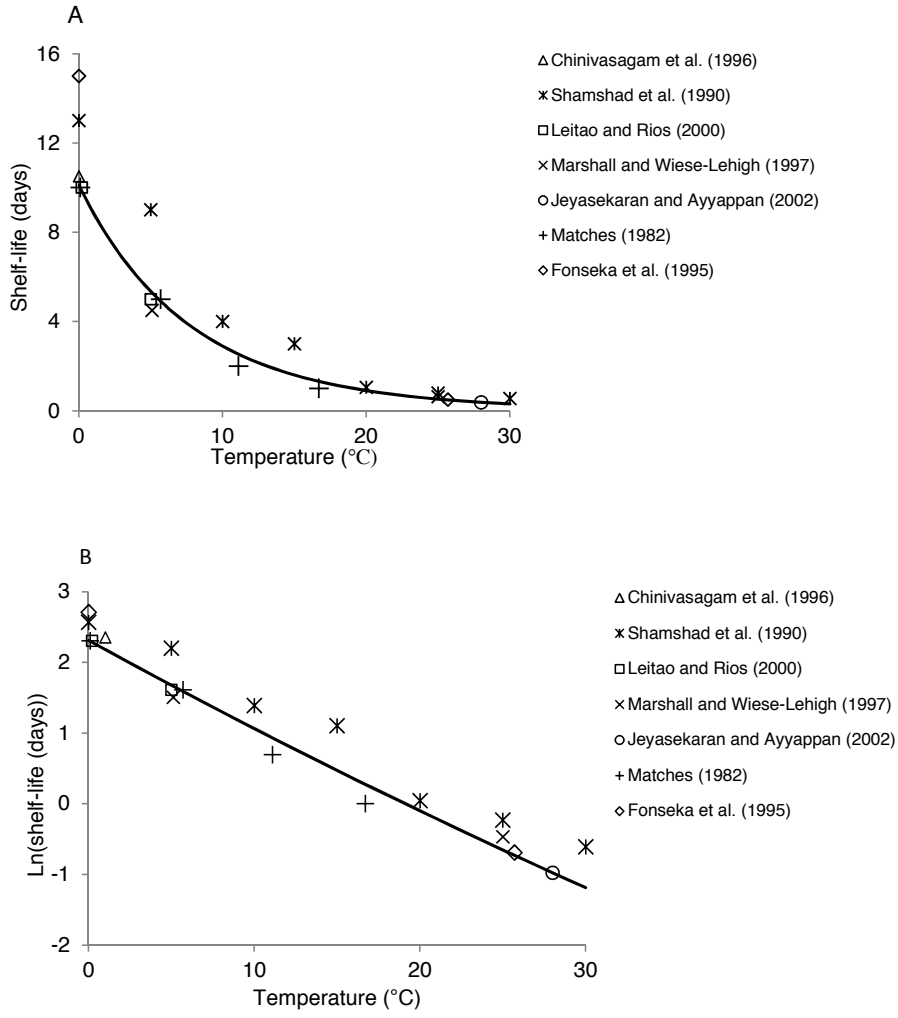

Fig. 7.1 Comparison of predicted shrimp shelf-life (solid line, Eq. (7.1)) and observed shrimp shelf-life in literature A = untransformed data, B = ln-transformed data.

The validation of this model in chapter 5 showed that it can be used to successfully predict the shelf-life of fresh tropical brackish water shrimps or cooked shrimps inoculated with *Pseudomonas psychrophila* stored at constant temperatures. In the present chapter, data from literature on shelf-life of various shrimp species were benchmarked to compare the outcomes of the model graphically (Fig. 7.1). The performance of the model was also assessed using the bias factor (B_f) and the accuracy factor (A_f) according to Ross (1996) as follows:

$$B_f = 10^{\left(\sum \log\left(t_{predicted}/t_{observed}\right)/n\right)} \quad (7.2)$$

$$A_f = 10^{\left(\sum \left|\log\left(t_{predicted}/t_{observed}\right)\right|/n\right)} \quad (7.3)$$

where $t_{predicted}$ are the predicted shelf-life values (days), $t_{observed}$ are the observed shelf-life values (days), n is the number of observations. As shown in Fig. 7.1, the model gives, overall, a successful prediction of shrimp shelf-life. The B_f value of 0.87 obtained also showed that successful prediction can be obtained from the model (Dalgaard, 2000). Thus, the developed model can be used as a tool in decision-making regarding the management of the quality of tropical brackish water shrimp as well as other shrimps caught or harvested worldwide and stored at constant temperatures.

However, this model can only predict shrimp shelf-life under constant temperature regimes and at the given initial number of spoilage organisms. Therefore, in chapter 5, new models were constructed based on the concentration of *Pseudomonas* spp. in shrimps and validated under dynamic temperature regimes simulating temperature fluctuation in the supply chain. In that chapter, the necessity of cooling shrimps as soon as they are caught and maintaining the cold chain along the supply chain was highlighted for better quality management.

The off-odor or off-flavor of spoiled fishery products is mainly caused by bacterial metabolism (Gram and Huss, 1996). A chemical compound (metabolite) that can be used as spoilage indicator may be useful to evaluate fishery products spoilage. In chapter 4 we found that total volatile basic nitrogen (TVBN) correlated well (R^2 >0.90) with fresh tropical shrimp spoilage and we concluded that it could be used as chemical spoilage indicator of *P. notialis*. TVBN is composed of ammonia (NH_3), dimethylamine (DMA), and trimethylamine (TMA). In crustaceans, NH_3 is the main component of TVBN (Vanderzant et al., 1973; Yeh et al., 1978) and its production results from deamination of amino acids (Herbert and Shewan, 1975). Bacteria associated with fishery products produced many other volatiles components that result in spoilage of the products. Using gas chromatography/mass spectrometry (GC/MS), Chinivasagam et al. (1998) found that amines, sulphides, ketones and esters were the main volatile components associated with spoilage of some shrimp species. The authors also found that closely related bacterial strains produced different volatile components from the same substrate. In this thesis, bacterial metabolites responsible for shrimp spoilage were not determined in detail. However, TVBN, that was found to be a good chemical indicator of shrimp in our study, is the most widely used parameter to evaluate the spoilage of fishery products according to Chan et al. (2006) and Pacquit et al. (2007). An advantage of using TVBN as chemical indicator of spoilage is that its determination is simple and does not require heavy equipment such as GC apparatus.

Since TVBN was found to be a good indicator of tropical shrimp spoilage, mathematical models predicting its production will be of relevance in shrimp quality management. It is known that TVBN production in shrimps is due to their tissue enzyme and microbial spoilage (Lopez-Caballaro et al., 2007, Mendes et al., 2002). Assuming that TVBN production follows a first-order reaction (Eq. (7.4)) (Labuza, 1984).

$$C = C_0 \exp(kt) \quad (7.4)$$

where t is the storage time (days), C is TVBN concentration at time t (mg/100 g), C_0 is the concentration of TVBN at time zero (mg/100 g) and k is the rate constant. The rate constant can be related to temperature using the Arrhenius' law (Eq. (7.5)).

$$k = A\exp\left(\frac{-E_a}{R\theta}\right) \quad (7.5)$$

with A is the pre-exponential factor, E_a is the activation energy (J mol^{-1}), R is the gas constant 8.314 (J mol^{-1} K^{-1}), θ is absolute temperature (K).

The efect of temperature on TVBN production can thus be predicted using Eq. (7.6)

$$C = C_0 \exp\left(A\exp\left(\frac{-E_a}{R\theta}\right)\cdot t\right) \quad (7.6)$$

or

$$\ln\left(\frac{C}{C_0}\right) = A\exp\left(\frac{-E_a}{R\theta}\right)\cdot t \tag{7.7}$$

Eq. (7.7) was fitted to TVBN production data obtained in chapter 4 from both lakes and the estimated parameters E_a and A were 85.0 kJ mol^{-1} and 1.6×10^{15} respectively. The E_a value obtained is similar to that obtained for shelf-life evaluation based on sensory analysis in chapter 4, which was 80.2±4.5 kJ mol^{-1}. This could be explained by the high correlation (R^2 >0.90) found between TVBN production and sensory scores. The E_a values obtained in this thesis, support earlier reports that the effect of temperature on the shelf-life of fishery products from warm waters corresponded to an E_a value of approximately 80.0 kJ mol^{-1}, which is higher than that of fishery products from cold or temperate waters (approximately 65.0 kJ mol^{-1}) (Dalgaard and Jorgensen, 2000).

To validate the model, TVBN values at different temperatures in chapter 5 were used and are presented in Fig. 7.2. To assess the performance of the model, B_f (Eq. (7.2)) and A_f (Eq. (7.3)) were also calculated. In this case, for each storage temperature, $t_{observed}$ are times (days) at which a given TVBN concentration was observed, $t_{predicted}$ are predicted times (days) to reach this same TVBN concentration. The B_f and A_f values are presented in Table 7.1. The B_f values were in the range of 0.75-1.25, which indicates a successful evaluation of seafood spoilage models according to Dalgaard (2000).

From Eq. (7.7), the shelf-life of shrimp can be predicted based on TVBN production. If the TVBN value at the sensory rejection time was set to 75 mg/100 g for whole fresh shrimps (average value obtained in this thesis), the shelf-life of a batch of shrimp with known initial concentration of TVBN, can be predicted using Eq. (7.8) (with the estimated values of E_a and A). The average initial TVBN values obtained in chapter 5 was used, i.e. 29.2 mg/100 g.

$$t = \frac{\ln\left(\frac{C}{C_0}\right)}{1.6\times10^{15}\exp\left(\frac{-1.02\times10^{4}}{\theta}\right)} \tag{7.8}$$

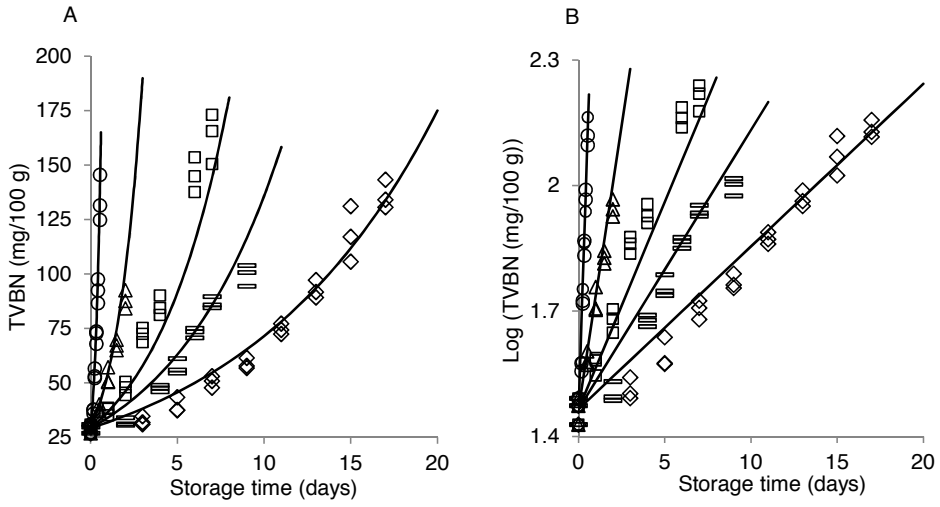

Fig. 7.2 Comparison of predicted TVBN values (solid line) and observed TVBN values during storage at 0ºC (◇), 4ºC (▭), 7ºC (□), 15ºC (△), and 28ºC (○). A = untransformed data, B = log-transformed data.

Table 7.1
Bias factor and accuracy factor of the model predicting TVBN production at constant storage temperatures

Storage temperature (°C)	Bias factor (B_f)[a]	Accuracy factor (A_f)[a]
0	0.92	1.12
4	0.86	1.17
7	1.12	1.17
15	0.90	1.12
28	0.85	1.19

[a]B_f and A_f were calculated for each storage temperature based on the comparison between the time to each observed TVBN value and the time predicted to reach the same TVBN value.

Eq. (7.8) can be used to determine shrimp shelf-life as well as the amount of time required to obtain a given TVBN value, which may indicate a given shrimp freshness level. For instance, based on the relationship between TVBN production and sensory analysis in our study on shrimp spoilage (chapters 4 and 5), the TVBN value of whole fresh brackish water tropical shrimp can be used to classify shrimps as shown in Table 7.2.

Table 7.2
Fresh tropical brackish water shrimp quality based on TVBN values

TVBN values (mg/100 g)	Shrimp quality
<30	High
30-40	Good
40-55	Acceptable
55-75	Marginal
>75	Unacceptable

Mendes et al. (2002) also suggested different degrees of freshness of deepwater pink shrimp (*Parapenaeus longirostris*) caught from Portuguese coast as follows: levels less than 30 mg/100 g for fresh shrimps, 30–40 mg/100 g edible with only slight decomposition, 40–60 mg/100 g as freshness borderline and above 60 mg/100 g for inedible and decomposed shrimps. It is known that TVBN values depend on fishery products species (Chinivasagam et al., 1996; Goulas and Kontominas, 2007). The method used to determine TVBN may also affect the obtained values (Botta et al., 1984). The difference in the set limits of TVBN could be explained by differences in species used (*P. notialis* versus *P. longirostris*) and determination methods applied (steam-distillation versus Conway microdiffusion).

Fishery products spoilage is a complex process. Therefore, it could be better to use a combination of diverse approaches for fishery products spoilage evaluation. In addition to microbiological aspects of spoilage, food microbiologists may integrate chemical spoilage. For instance, a relationship between the production of the chemical indicator of spoilage and the increase in number of the SSO through mathematical models would be of relevance to fishery products quality management. If validated, these models can be used to estimate the concentration of SSO from the determined values of the spoilage indicator or vice-versa. Data on growth kinetic of *Pseudomonas psychrophila* and TVBN production in cooked shrimp obtained in chapter 5 were used in this chapter as illustration. For each storage temperature (0, 4, 7 and 15°C), a simple mathematical equation was used (Eq. (7.9))

$$TVBN(t) = a\Delta N + TVBN(0) \quad (7.9)$$

Where *TVBN(t)* is TVBN value (mg/100 g) at time *t* (in days), *TVBN*(0) is the TVBN at time zero, *ΔN* is absolute increase in number of microorganism at time *t* (log CFU/g), and *a* is the parameter to be estimated. Regression analysis of the different values of *a* and the different temperatures showed a good fitting (R^2 =0.99) with polynomial function and the parameters were estimated (Eq. (7.10))

$$TVBN(t) = \left(0.011T^2 - 0.376T + 9.3\right)\Delta N + 10.8 \quad (7.10)$$

where T is the temperature (degree Celsius).

Eq. (7.10) implies that TVBN production derives only from microbial growth. However, shrimp tissue enzymes can also contribute to TVBN production (Lopez-Caballaro et al., 2007, Mendes et al., 2002).

In addition to provide complementary results to microbiological results, chemical aspects of spoilage provide results more rapidly. For example, in developing countries such as Benin where microorganisms enumeration is mainly based on the conventional plate count method, for a given shrimp sample, it will take 48-72 h to obtain microbiological results, while the TVBN value of the same sample can be obtained in 1-2 h. Clearly, fishery products spoilage prediction software that combines microbiological and chemical evaluation of spoilage will be of more interest for fishery products community than software that addresses only microbiological spoilage.

Sensory analysis is the key analysis in fishery products freshness evaluation. Indeed, consumer acceptance of fishery products is determined by sensory perception (Huidobro et al., 2000). Also, the sensory rejection time is often used to determine the microbiological or chemical spoilage level i.e. the microbial or chemical concentration reached when the product spoils. Therefore, care must be taken when performing sensory analysis of fishery products. Panelists employed in laboratories or fishery products industries should be well trained to reduce the subjectivity inherent in sensory analysis. Also, the quality parameters and their descriptions should be clearly established. To that end, the Quality Index Method (QIM) can be useful since the method describes precise, objective and independent quality descriptors that are used to standardize sensory assessment for each fishery products species (Luten and Martinsdottir, 1997; Olafsdottir et al., 2004). Although the QIM scheme has been developed for several fishery products species (Sant'Ana et al., 2011), it has not yet been developed for tropical brackish water shrimp *P. notialis*. Future studies should address this aspect.

Fishery products safety management

For a given fishery product, pathogens identification and the categorization of their risks (risks ranking) are of importance to efficiently manage the risks associated with the consumption of that product. Hazard identification in risk assessment or hazard analysis critical control points (HACCP) has been discussed by several authors (Bassett et al., 2012; Janevska et al., 2010; Lammerding et al., 2001; Van Gerwen et al., 1997). From these studies, hazards can be identified from published literature, epidemiological studies, foodborne disease reports, or expert opinion. However, some hazards may be overlooked since epidemiological studies, foodborne disease reports, and even published literature often target well-recognized foodborne pathogens associated with a given product. For example, *Bacillus cereus* is not on the list of indigenous bacteria associated with fishery products according to Amagliani et al. (2012), Feldhusen (2000), and Huss (1997). However, using a molecular method (clone library analysis) in chapter 3, *Bacillus cereus* was detected in high proportion (44% of the clones, n=55) in one of our clone libraries obtained from freshly caught shrimps in brackish waters in Benin (West Africa). Therefore, detection of the pathogens in some cases may be required to have a complete picture of the hazards to be identified for risk analysis or HACCP. Given the variability inherent in the ecology of fishery products from brackish waters (for example water salinity ranges between ca 0 to 30‰ depending on the season and sampling point), the detection of pathogens associated with fishery products or their environment should take into consideration spatio-temporal variation. Pathogens detection in fishery products by combining culture-dependent and culture-independent approaches can also reveal emerging foodborne pathogens that could be considered in risk analysis or HACCP at least for vulnerable people (aged, infants, immuno-depressed). For example, *Lactococcus garvieae* detected in shrimps both by culture-dependent and culture-independent methods in this thesis (chapters 3 and 4) is a well-recognized fish pathogen, which is also responsible for clinical cases in humans, especially, aged people and people with gastrointestinal disorders (Aguado-Urda et al., 2011; Wang et

al., 2007). The organism has been related to human septicemia, infective endocarditis, urinary and skin infections (Chan et al., 2011; Wang et al., 2007).

For microbiological hazards, a risk is defined as the product of the probability of illness due to a particular microorganism in a certain food, and the severity of illness, in a specific group of consumers (Reij and Van Schothorst, 2000). As it is defined, not all the pathogens associated with a food will pose the same risk to consumers. Microbiological risks associated to the product can therefore be categorized (risk ranking). Risk ranking is an important tool which can help in prioritizing the management of risks associated with a given product (Da Silva Felicio et al., 2015; Van Asselt et al., 2012). Risk ranking tools available for risk assessment have been reviewed (Bassett et al., 2012) and in chapter 6, we also used a simple mathematical model described by Zwietering (2005) (Eq. (6.1)) to categorize the risks posed by the identified pathogens in a given food product. The main problem with the use of all risks ranking tools is the limitation in available data, which leads to many assumptions (Reij and Van Schothorst, 2000). Risk assessors should be aware that in risks ranking some assumptions are more crucial than others. For instance, a poor estimation of the number of servings or the mass of a serving in the population although it will affect the risk estimate for each pathogen, will have no impact on the categorization of the risks posed by the pathogens since this factor is common to all the pathogens. Poor estimations of factors specific to each pathogen such as its prevalence in the raw product, its concentration in the food and the dose-response parameter of the pathogen will lead to a wrong ranking of the foodborne pathogen risks.

Once the microbiological risks associated with the consumption of foods are assessed and categorized, the next step is the management of the main pathogens risks. The Food Safety Objective (FSO) approach can be used to link food risk assessment to risk management (ICMSF, 2002). An FSO specifies the level of a hazard that is considered tolerable in the final product for public health protection. It often derives from the so-called appropriate level of protection (ALOP) (Gorris, 2005; Zwietering, 2005). The ALOP, expressed for instance as the number of illnesses in a population per annum, defines on a population level what level of risk a society is prepared to tolerate or is considering to be achievable (Gorris, 2005). When an ALOP for a given population is known, mathematical equations, as demonstrated in chapter 6 can be used to determine the FSO. To meet the FSO in the final product, one can set performance objectives (PO) at earlier steps in the supply chain. The FSO approach can therefore be used as an integral part of food chain management by for example, assigning responsibilities over the various parts of the food chain (Gorris, 2005; Zwietering, 2005).

In addition to foodborne pathogens, chemical hazards are a matter of concern to fishery products safety as well. Fish species that carry toxins are the most incriminated. The two most common types of toxins are ciguatoxin and scombrotoxin (histamine poisoning) (Huss et al., 2000; Reames, 2012). Ciguatoxin fish poisoning (ciguatera) can occur when food is prepared from fish harvested in subtropical and tropical waters where toxic algae are consumed by small reef fish, which are eaten by larger reef fish that are then consumed by humans (Lum, 2012). Histamine poisoning on the other hand, is caused by abusive handling of scombroid fish species (Huss et al., 2000).

Heavy metals and pesticides from polluted fishing areas are chemical hazards that can contaminate fishery products. Cadmium, lead and mercury are regarded as the most toxic metals in fishery products (Marques et al., 2010). They are causative agents of various adverse health effects, including neurological, nephrological and immunological damage, endocrine system dysfunctions and carcinogenic and teratogenic activities (Cirillo et al., 2010). In countries where pesticides are intensively used in agriculture, run-off of rain and agricultural irrigation water introduce them into the aquatic environment, where they pose potential health risks to fishery products and to humans consuming them (Castillo et al., 1997; Scott et al., 1990).

Moreover, fishery products contain allergens that cause adverse reactions to the fraction of the consuming population that is allergic to specific fishery products (Taylor and Nordlee, 1993; Tsabouri et al., 2012). The risk of an allergic reaction from the consumption of shrimps is particularly high in some people (Flick and Granata, 2010). The allergens are mainly proteins naturally present in fishery products that can persist

and cause allergy to some people even after rigorous treatment like cooking and extreme pH (Taylor and Nordlee, 1993; Tsabouri et al., 2012). In fish, parvalbumin, a calcium-binding sarcoplasmic protein, is the most important allergen whereas in shellfish, including shrimp, tropomyosin, a muscle protein, is the major allergen. In addition to the naturally occurring proteins responsible for allergic reactions, some additives, especially sulfite agents used to control melanosis (black spot) in crustaceans (mainly shrimps) can also provoke allergic reactions to some consumers (Slattery et al., 2009; Taylor and Nordlee, 1993).

In this thesis, chemical hazards associated with tropical brackish water shrimps were not studied. We addressed foodborne pathogens commonly ranked by food safety scientists as the greatest hazards from the consumption of fishery products (Taylor and Nordlee, 1993). In fact, risk from chemical contaminants such as heavy metals and pesticides in marine fishery products, is relatively low (Huss et al., 2000). But the risk from heavy metals and pesticides contamination of fishery products from brackish waters may be higher. However, in Benin, the analyses of heavy metals carried out in 2007 on shrimps from Lake Aheme revealed that the concentrations of lead, cadmium and mercury were all lower than the allowed limits (Coopération Belge au Dévelopement, 2007). In Lake Nokoue, except for lead whose concentration exceeded the allowed limits in some samples, cadmium and mercury concentrations were lower than the tolerable limits in all samples analyzed (Aina et al., 2012). However, the fact that the limits set are not exceeded should not be considered as sufficient evidence of food safety because of uncertainties related to the toxicological assessment of contaminants (Marques et al., 2011). The management of chemical risks associated with fishery products should be of concern. For shrimps in Benin, research is needed on the concentration of heavy metals and pesticides in shrimps, water and sediment taking into account the variability in fishing areas and shrimp catching seasons. This will enable the authorities in charge of fishery products to restrict shrimp catching in the fishing areas with high chemical contamination levels. A well designed monitoring system to control the levels of chemical contaminants in other fishing areas as well as in shrimps caught from that areas should be implemented. Moreover, people around the lakes should be trained in good waste management to avoid dumping domestic waste into the lakes. As for melanosis control, to reduce allergic reactions due to shrimp consumption, the use of substitutes for sulfite should be investigated. 4-Hexylresorcinol, catechin and ferulic acid are some compounds proved to efficiently prevent melanosis formation in some shrimp species namely *Parapenaeus longirostris* and *Penaeus vannamei* (Montero et al., 2004; Nirmal and Benjakul, 2010). However, the effective dose of antimelanosic compounds varies according to fishery products species and postharvest conditions (Otwell, 2008). Therefore, research is needed on the possibilities of using other antimelanosic compounds than sulfite to prevent melanosis in *P. notialis*.

Fishery products supply chain

A supply chain is an integrated process wherein a number of various business entities work together in different processes and activities to bring products and services to the market, with the purpose to satisfy customers' demands (Beamon, 1998; Christopher, 2005). Fishery products may be supplied to a wide range of intermediate and final destination customers (Lim-Camacho et al., 2014). The Beninese export-oriented shrimp supply chain described in chapter 2 of this thesis (Fig. 2.2) involves shrimp fishers, intermediate traders 1, intermediate traders 2, collectors and processors who also export shrimps to Europe. This shrimp supply chain is similar to the Vietnamese shrimp supply chain in which shrimp farmers sell shrimps to collectors who in turn sell shrimps to middle traders, who in turn sell their shrimps to wholesale agents, who sell shrimps to processors (Tran et al., 2013). In such a supply chain, shrimps are exposed to various hands, holding containers, in conditions where the cold chain is not always well maintained. Therefore, the risk of affecting shrimp quality and safety is high. For perishable food commodities such as fishery products, their quality and safety can be better assured with short supply chains. Beninese export-oriented shrimp supply chain could be reorganized so that shrimp collectors use isothermal containers with ice provided by shrimp processors to buy shrimps directly from shrimp fishers and

transport them to shrimp plants. In this supply chain, the relationships between stakeholders (e.g. family relationship), that pose a threat to shrimp quality and safety as discussed in chapter 2 will also be reduced. Of course, some stakeholders, especially intermediate traders will have to find alternative employment in this new supply chain, but overall, the efficiency of the chain, the quality and safety of the products can increase. As a result, shrimps may be bought from shrimp fishers at higher price and some stakeholders, especially the shrimp fishers' wives can benefit from that.

In addition to quality and safety issues, consumers are increasingly interested in the origin of their food and the sustainability of the processes used to produce and to deliver it (Wognum et al., 2011). To that end, information systems based on identification, registration, tracking and tracing capabilities through traceability in food supply chains are needed (Trienekens and Van der Vorst, 2006). A good traceability system in the Beninese shrimp supply chain can for example make information available related to the types of fishing nets to catch shrimps. Some stakeholders may avoid buying shrimps from fishermen who use fishing nets with fine meshes (see Table 2.2) because this practice cannot assure the sustainability of shrimp catching from the lakes.

Our investigation in the shrimp supply chain (chapter 2) highlighted the necessity of training in Good Fishing Practices and safe food handling practices to improve shrimp quality and safety. This training should be a continuous process. Local radio stations, non-governmental organizations, and agricultural extension services can be used for that purpose.

Fishery products in international trade

Global fishery products trade is increasing with the vast majority of fishery products exported by developing countries to developed countries (Baylis et al., 2011). For example, about 75% of shrimp production, whether cultured or wild-caught, originates from developing countries, but 70 to 75% of global shrimp consumption occurs in developed countries (Flick and Granata, 2010). Consumers, especially in developed countries have become more aware of the impact of food production, processing, and distribution on public health and they are demanding food products of high and consistent quality (Trienekens and Zuurbier, 2008; Wognum et al., 2011). To meet the expectations of their consumers, public and private authorities in developed countries have been establishing more and more stringent food safety standards. Unfortunately, developing countries have difficulties to meet some of these standards. As a result, access to international market can be denied to them. For example, several bans or self-bans on export of fishery products from Asian and African countries to Europe have been reported (Alavi, 2009; Dabadé et al., 2014; EEC, 1998; Golnaz et al., 2012; Henson and Mitullah, 2004; Ponte, 2007) with detrimental consequences on their economy.

The question is whether the imposed ban or self-ban is the best approach to be adopted. In the author's view, it is a good idea that fishery products importing countries establish food safety standards and require exporting countries to meet them as they do. In fact, all consumers (including those from exporting countries) will benefit from the fulfilment of the standards through the reduction of the adverse health effects due to the consumption of the products. However, one should realize that the products are not systematically unsafe when all standards are not met. While the authorities in developing countries are making all the necessary to meet food safety standards, end-product testing -although costly- can be intensified for their products.

As reported by several authors, poor infrastructure, limited institutional capacity and lack of skilled people are making it difficult for developing countries to meet standards and to integrate into global markets (Martinez and Poole, 2004; Trienekens and Zuurbier, 2008; Van Veen, 2005). Institutions such as the World Bank, FAO, WHO and developed countries should assist developing countries to comply with food safety standards. For fishery products in particular, developed countries may be obliged to give this assistance since their needs in fishery products are provided mainly by developing countries.

Overall conclusion

Through a wide-chain survey, this thesis describes technical aspects of relevance for better management of shrimp quality and safety in a developing country that exports its shrimps to developed countries. The thesis contributes to the knowledge about tropical shrimp spoilage. Using quantitative studies, models predicting the shelf-life of tropical shrimps under constant storage temperatures or under dynamic temperature regimes simulating temperature fluctuation in the supply chain were constructed and validated. The relevance of combining microbiological, chemical and sensory analyses to evaluate food spoilage was discussed. In addition to shrimp quality aspects, the thesis assessed microbiological risks associated with the consumption of shrimps using different tools. Moreover, the thesis provides insight into freshly caught tropical brackish water shrimp (*P. notialis*) microbial diversity and concentration in relation to their ecology.

Finally, other relevant aspects of shrimp safety not studied in thesis namely chemical contaminants were addressed and suggestions for their study in future research were made. Although this thesis deals with shrimp in one specific developing country, the approaches used, the tools and strategies developed can be applied or adapted for a better management of quality and safety of other fishery products in other developing countries.

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Summary

Shrimps constitute one of the most important fishery products exported from developing countries to the developed world. However, shrimps spoil fast and they may also be a source of foodborne pathogens. To meet the expectations of consumers who are increasingly interested in high quality food, shrimp quality and safety management is of great importance. In Benin (West Africa), shrimps are caught from brackish waters (lakes and lagoons), and are processed and sold in the domestic market or exported to Europe mainly as raw frozen. To contribute to a stable access of Beninese shrimps to the international market technical and microbiological aspects of shrimp quality and safety management are described in this thesis.

Knowledge of the types of bacteria associated with shrimp and their initial concentrations is of importance to develop strategies and tools for a better shrimp quality and safety management. Therefore, we combined culture-dependent (plate counts) and culture independent (DGGE, clone libraries analysis) approaches to determine the diversity and load of bacteria associated with freshly caught tropical shrimps as well as that of their habitats (sediment and water) in different shrimp fishing periods. In shrimp samples, aerobic mesophilic counts (reported as total viable counts (TVC), ranged between 4.4 and 5.9 log CFU/g. In general, the level of the groups of microorganisms enumerated was lower in sediment and water samples and even lactic acid bacteria (LAB) were below the detection limit in sediment and water samples, as were *Enterobacteriaceae* in sediment samples. As for diversity, in shrimp samples, Gram-positive bacteria were mainly represented by LAB, *Staphylococcus* spp., *Macrococcus* spp., *Bacillus* spp., *Clostridium* spp. and *Corynebacterium* spp., and Gram-negative bacteria, mainly by *Enterobacteriaceae*. Bacterial diversity was much higher in sediment and water samples. Moreover, our results showed that at species level, distinct bacterial communities were associated with sediment, water or shrimp samples. In fact, among water, sediment and shrimp sampled at the same site the same day, only one species (uncultured *Xanthomonadales*) was shared between the bacterial community of water and that of sediment. No species from shrimp microbial community was shared neither with those of sediment nor water. Thus, it seems that the bacterial community of tropical brackish water shrimps cannot be predicted from the microbiota of their aquatic environment.

To obtain insight in the factors that determine shrimp (microbiological) quality and safety, the whole supply chain was surveyed. The steps of the chain prior to shrimp processing at the plants were critical for shrimp quality and safety because of prevailing temperature abuse and inappropriate hygienic conditions. For example, freshly caught shrimps were kept at ambient temperature (28°C) up to 6 h by stakeholders and were often stored in inappropriate holding containers, and were sometimes washed with non-potable water. Shrimps handled under these conditions will allow the proliferation of bacteria that cause spoilage (quality issue) or pose health risks to consumers (safety issue). It is known that not all microorganisms associated with freshly caught fishery products contribute to their spoilage. To identify the microorganisms that contribute mainly to the spoilage of shrimps from tropical brackish waters, experiments were performed combining microbiological, chemical and sensory analyses of fresh shrimps (*Penaeus notialis*) to evaluate their spoilage characteristics at different storage temperatures. The spoilage microbiota of *P. notialis* was storage temperature-dependent. During storage in ice (0ºC), *Pseudomonas* spp. were dominant, whereas at 7ºC and 28ºC H_2S-producing bacteria were the dominant group of microorganisms. A good correlation (R^2>0.90) was obtained between the chemical parameters determined, especially total volatile basic nitrogen (TVBN) or trimethylamine (TMA) and the sensory scores. An empirical model to predict the shelf-life of naturally contaminated tropical shrimp as a function of storage temperature was developed. To find out which bacteria were mainly causing spoilage, specific groups of organisms were isolated at the sensory rejection times and assessed for spoilage potential in sterile shrimps. Isolates capable of producing strong off-odor were identified by 16S rRNA sequencing as mainly LAB and *Enterobacteriaceae* at 28ºC or 7ºC and *Pseudomonas* spp. and LAB (*Carnobacterium maltaromaticum*) at 0ºC. Potential spoilage-causing LAB were isolated from the H_2S-producing bacteria group.

The empirical shelf-life prediction model developed could only predict shrimp shelf-life under constant temperature regimes and at the given initial number of spoilage organisms. To further develop the model, we first conducted a growth experiment with the isolates capable of producing strong off-odor in shrimps at 7°C (maximum temperature at which shrimps should be stored to be accepted by shrimp processors in Benin). The fastest growing isolates of the fastest growing groups of microorganisms, namely, *Pseudomonas psychrophila* (from the pseudomonads) and *Carnobacterium maltaromaticum* (from the LAB), were selected for their spoilage activity in sterile shrimps and for modeling studies. *P. psychrophila* had a higher growth rate and a higher spoilage activity at 0 to 15ºC, while at 28ºC, *C. maltaromaticum* had a higher growth rate. Models predicting the growth of *P. psychrophila* in sterile shrimps and *Pseudomonas* spp. in fresh naturally contaminated shrimps as a function of temperature were constructed. The validation of these models under dynamic storage temperatures simulating actual temperature fluctuation in the shrimp supply chain showed that they can be used to predict the shelf-life of cooked and fresh tropical shrimps.

As far as food safety is concerned, we identified potential microbiological hazards associated with tropical brackish water shrimps. The risks associated with these hazards to the consumers in the importing countries were classified using different approaches. The results showed that overall, the main foodborne pathogen risks were from *Vibrio parahaemolyticus* and *Salmonella*. However, the risks from viruses and pathogenic *Escherichia coli* were also high. Quantitative microbiology was used to predict the growth of *V. parahaemolyticus* and *Salmonella* under dynamic storage temperatures simulating temperature fluctuation in the shrimp supply chain as well as their inactivation under various temperatures. The management of the risks posed by the main pathogens was addressed using different scenarios to meet the set food safety objectives.

In conclusion, this thesis showed that the steps of fishing, collection and transport of shrimps to plants are the main steps in the Beninese shrimp supply chain that require special care to improve shrimp quality and safety. It demonstrated that the overlap between shrimp microbiota and that of their surrounding (water, sediment) is low. The specific spoilage organism of *P. notialis* was found to be *Pseudomonas. psychrophila* at low storage temperatures. At high storage temperatures (>7°C) however, LAB and *Enterobacteriaceae* were the main spoilage organisms. The main foodborne pathogens risks were from *V. parahaemolyticus* and *Salmonella*. Mathematical models predicting shrimp shelf-life were developed. Their validation showed that such models can be used as a decision support tool for shrimp quality management.

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Sylvain

List of publications

Dabadé, D.S., Azokpota, P., Nout, M.J.R, Hounhouigan, D.J., Zwietering, M.H., den Besten, H.M.W., 2015. Prediction of spoilage of tropical shrimp (*Penaeus notialis*) under dynamic temperature regimes. International Journal of Food Microbiology 210, 121-130.

Dabadé, D.S., den Besten, H.M.W., Azokpota, P., Nout, M.J.R., Hounhouigan, D.J., Zwietering, M.H., 2015. Spoilage evaluation, shelf-life prediction, and potential spoilage organisms of tropical brackish water shrimp (*Penaeus notialis*) at different storage temperatures. Food Microbiology 48, 8-16.

Adekambi, S., **Dabadé, D.S.**, Kindji, K., den Besten, H.M.W., Faure, M., Nout, M.J.R., Sogbossi, B., Ingenbleek, P.T.M. Towards a stable market access for the Beninese shrimp chain to the EU: quality, legal and marketing. In: Bijman, J., Bitzer, V. (Eds.), Co-Innovation and Quality in African Food Chains Wageningen, the Netherlands: Academic Publishers. In press.

Dabadé, D.S., den Besten, H.M.W., Azokpota, P., Nout, M.J.R., Hounhouigan, D.J., Zwietering, M.H., 2014. Quality perceptions of stakeholders in Beninese export-oriented shrimp chain. Journal of Food Protection 77, 1642-1648.

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Eteka, C.A., Ahohuendo, B.C., Ahoton, L.E., **Dabadé, D.S.**, Ahanchede, A., 2010. Seeds' germination of four traditional leafy vegetables in Benin. Tropicultura 28, 148-152.

Dabadé, D.S., Wolkers-Rooijackers, J.C.M., Azokpota, P., Hounhouigan, D.J., Zwietering, M.H., Nout, M.J.R., den Besten, H.M.W. Bacterial communities and diversity in fresh tropical shrimps (*Penaeus notialis*) and the surrounding brackish waters and sediment. Submitted for publication.

Dabadé, D.S., Hazeleger, W.C., Hounhouigan, D.J., den Besten, H.M.W., Zwietering, M.H. Microbiological risks classification and safety management of tropical brackish water shrimp (*Penaeus* spp.). Submitted for publication.

Curriculum vitae

D. Sylvain Dabadé was born on September 25, 1983 in Savalou, Republic of Benin (West Africa). He graduated from the secondary school in 2002 and was awarded the Beninese Government excellence grant for studying at the University of Abomey-Calavi (Benin). At the University, he was enrolled at the Faculty of Sciences and Technology (FAST/UAC) and the Faculty of Agronomic Sciences (FSA/UAC). He graduated in December 2008 as Agricultural Engineer with a specialization in Crop Sciences. In 2009, he was awarded a scholarship from Belgium ("CUD bourse locale") to enroll in an MSc program at the University of Abomey- Calavi. He achieved the Master degree in the field of Food Safety in 2010. The same year, he was granted a PhD scholarship from the Netherlands Universities Foundation For International Cooperation (NUFFIC), under the project NPT/BEN/263 to undertake his PhD project at the Laboratory of Food Microbiology at Wageningen University (the Netherlands). His PhD project was entitled: "shrimp quality and safety management along the supply chain in Benin". He carried out the research presented in this thesis from August 2010 to May 2015, alternately in Benin and Wageningen University.

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Courses

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Molecular Food Microbiology (2013), KVL, Copenhagen, Denmark
Genetics and Physiology of Food-associated Microorganisms (2013), VLAG, Wageningen

Meetings

Third colloquium of the University of Abomey-Calavi, Benin (2011) (oral presentation)
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Other activities

Preparation of PhD research proposal (2010)
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PhD study trip to Japan (2012)
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