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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 28 °C, 7 °C and 0 °C were assessed. H_2S-producing bacteria were the dominant group of microorganisms at 28 °C and 7 °C whereas *Pseudomonas* spp. were dominant at 0 °C. 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 28 °C or 7 °C and *Pseudomonas* spp. and LAB at 0 °C. 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.

1. 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 (0–10 °C) and *Vibrio* spp. at high storage temperatures (15–35 °C) (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

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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.

2. Materials and methods

2.1. Study with naturally contaminated shrimp

2.1.1. 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 0 °C, 7 °C and 28 °C. For 0 °C 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 4 °C. The ratio of shrimp to ice was approximately 1:3. Shrimps were re-iced daily to maintain the same ratio. For 7 °C and 28 °C 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.

2.1.2. 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 25 °C 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 (Martínez-Alvarez et al., 2009; Mai et al., 2011; Broekaert et al., 2011; Giuffrida et al., 2013). *Enterobacteriaceae* were enumerated on double-layered plates of violet red bile glucose (VRBG) medium and incubated at 37 °C for 24 h. *Pseudomonas* spp. were enumerated on spread plates of *Pseudomonas* agar base supplemented with cetrimide, fucidin, and cephaloridine (CFC) at 25 °C for 48 h. Lactic acid bacteria (LAB) were enumerated on double-layered plates of de Man, Rogosa and Sharp agar (MRSA) incubated at 30 °C 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.

2.1.3. 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 (Commission regulation (EC) No 2074/2005). 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 distillation ended after exactly 10 min, allowing the same distillation rate for all the samples. The volatile base components absorbed by boric acid solution (3%) in laboratory standard glassware (beaker, flask) 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).

2.1.4. 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 (Dalgaard et al., 1993; Mejlholm et al., 2008; Argyri et al., 2010) 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.

2.1.5. 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 (1) Ratkowsky model (2) (Ratkowsky et al., 1982) and Arrhenius model (3) were fitted to the combined RS-data.

$$RS = b_1 \times \exp(a \times T) \quad (1)$$

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

$$RS = b_3 \times \exp\left(\frac{-E_a}{R \times K}\right) \quad (3)$$

With T, the storage temperature (°C); R, the gas constant 8.314 (J mol^{-1} K^{-1}); K, temperature in Kelvin. b_i, a, T_{min} and E_a are 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).

$$RMSE_{model} = \sqrt{\frac{\sum (observed_i - fitted_i)^2}{n - s}} \quad (4)$$

With observed$_i$, observed values; fitted$_i$, described values; n, number of data points; s, number of parameters of the model.

2.2. 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 (Zheng et al., 2012; Rodríguez-Calleja et al., 2012). Pure cultures of isolated colonies were inoculated to freshly caught shrimp of which the endogenous flora was heat inactivated at 100 °C for 30 min. The inoculation was approximately 10^5 CFU/g of shrimp. The samples were stored as described in 2.1.1 at the temperature of initial isolation. The ability of the inoculated culture to produce off-odor was evaluated by the same panelists as described in 2.1.4. Strains deemed to produce strong off-odor by at least 50% of the panelists were identified following the procedure described below.

2.3. 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 94 °C for 5 min, then 35 amplification cycles with denaturation at 94 °C for 20 s, primer annealing at 56 °C for 20 s, and extension at 72 °C for 1 min, finally an extension at 72 °C for 7 min followed by cooling at 4 °C. 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.

3. Results

The actual storage temperatures recorded were 27.8 ± 0.4 °C; 7.1 ± 0.3 °C, and 0.1 ± 0.3 °C during storage at 28 °C, 7 °C, and 0 °C 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.

3.1. 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. 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).

During storage at 28 °C, 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

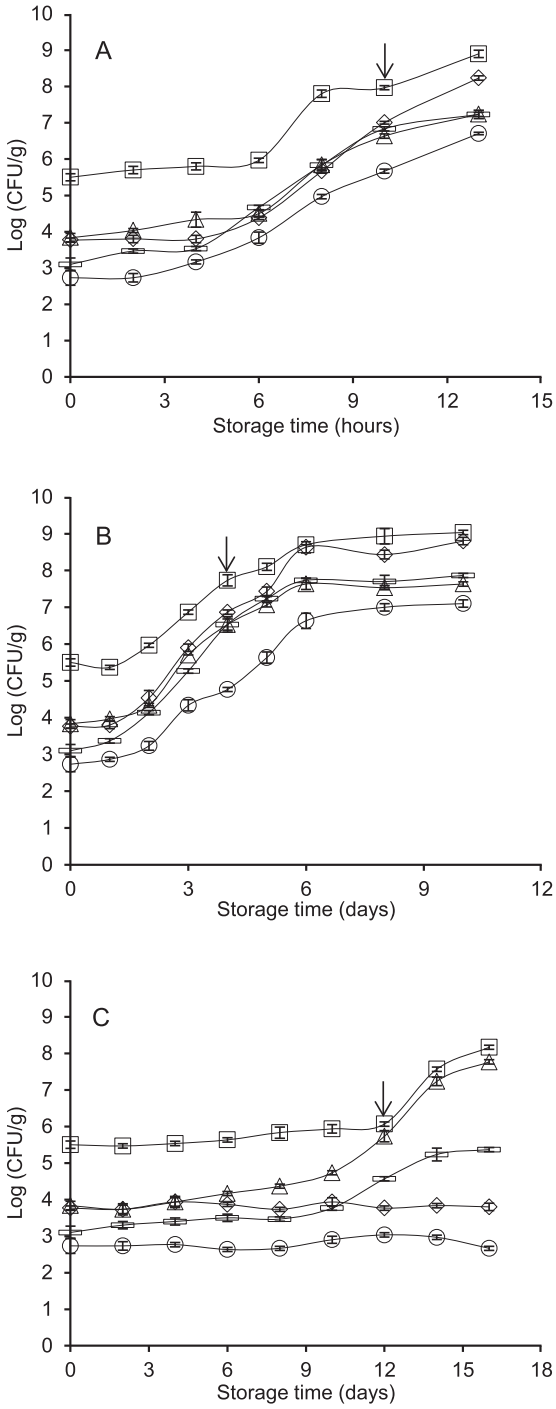

Fig. 1. Evolution of total viable counts (□), H_2S-producing bacteria (◊), *Pseudomonas* spp. (△), Enterobacteriaceae (○), and lactic acid bacteria (▭) of shrimp stored at 28 °C (A), 7 °C (B), and 0 °C (C). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent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

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 7 °C was similar but slower than the one observed at 28 °C. 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 7 °C 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 0 °C, 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 0 °C. 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.

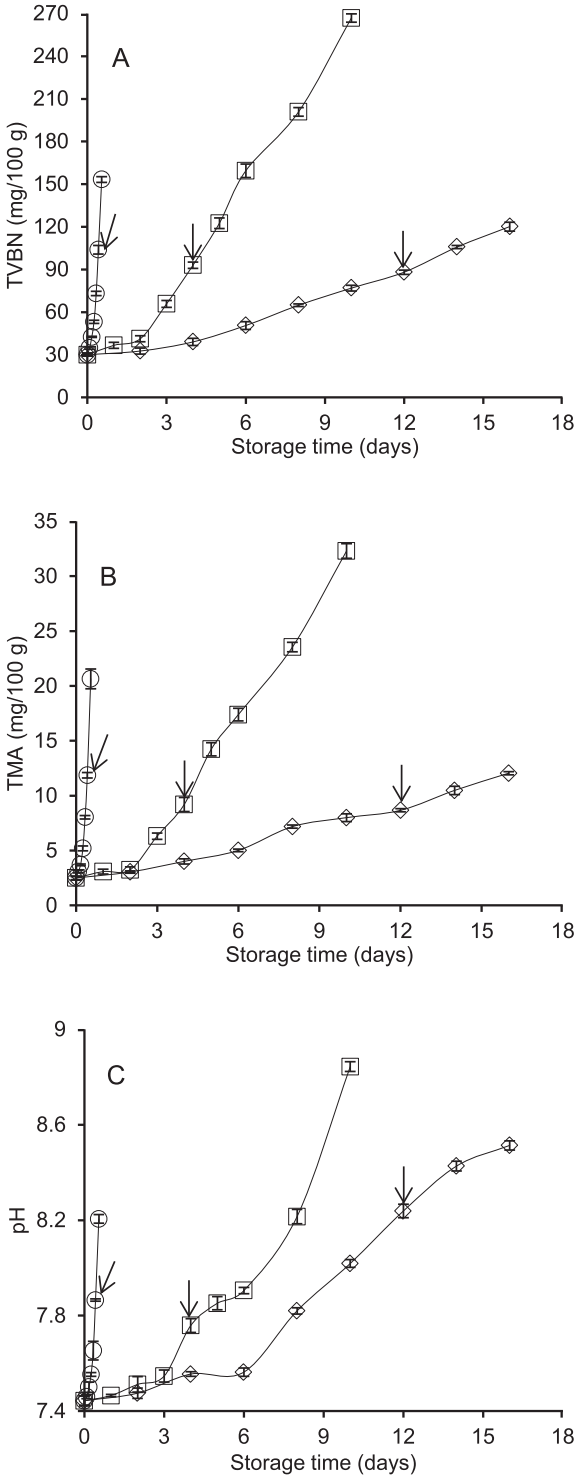

Fig. 2. Evolution of total volatile basic nitrogen (TVBN) (A), trimethylamine (TMA) (B), pH (C) of shrimp stored at 28 °C (○), 7 °C (□), and 0 °C (◊). Arrow indicates sensory rejection time. Bars represent the standard deviation of three independent samples.

3.2. Chemical changes

The evolution of TVBN, TMA and pH values in shrimp samples stored at 28 °C, 7 °C, and 0 °C is depicted in Fig. 2A, B and C 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 to 104 mg/100 g and TMA values, from 9 to 12 mg/100 g.

3.3. Sensory analysis

The results of shrimp sensory evaluation are presented in Table 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 28 °C, 4 d at 7 °C, and 12 d at 0 °C.

Table 1
Shrimp off-odor evaluation (in % of panelists) during storage at 28 °C (A), 7 °C (B), and 0 °C (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.

3.4. 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 by the 10 panelists at each sensory session (Fig. 3A and B). The correlation between pH values and the sensory scores was lower (Fig. 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).

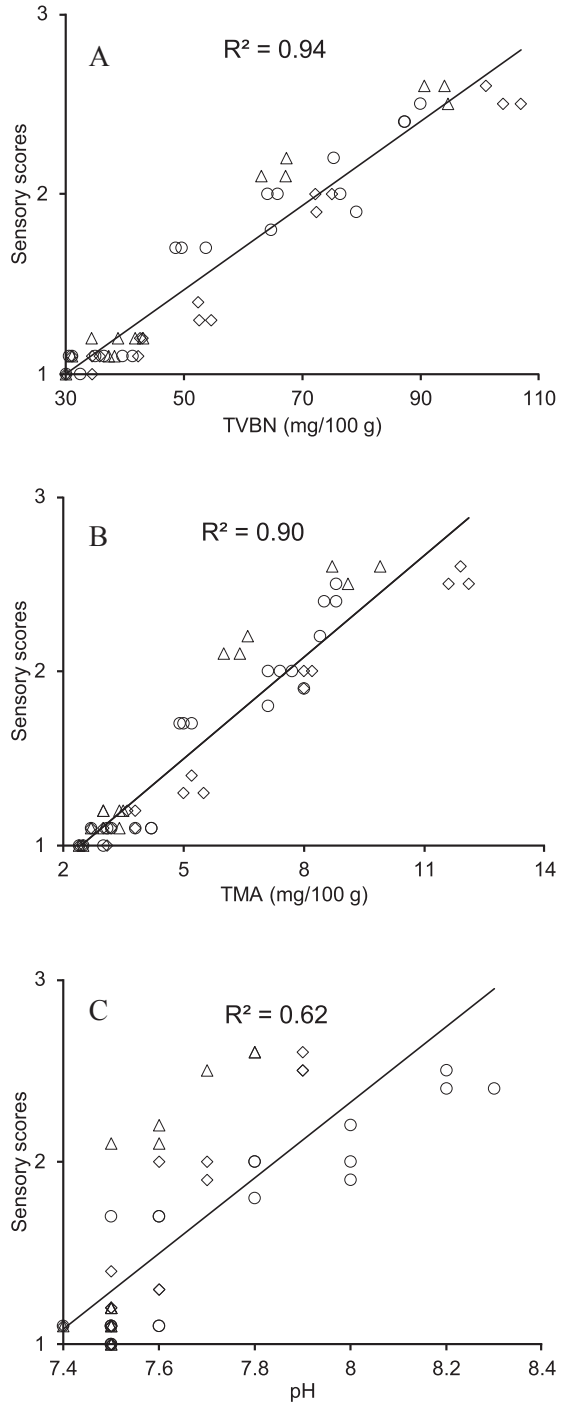

Fig. 3. Relationship between sensory scores and total volatile basic nitrogen (TVBN) (A), trimethylamine (TMA) (B), and pH (C) values from the beginning of shrimp storage at 28 °C (◊), 7 °C (Δ) and 0 °C (○) until the sensory rejection time. Sensory scores: 1 = shrimp without any off-odor, 2 = shrimp having slight off-odor but still being acceptable and 3 = shrimp producing strong off-odor. The average of 10 panelists was used.

3.5. Effect of temperature on spoilage rate and shelf-life prediction

The root mean square error ($RMSE_{model}$) 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.

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 equation (5)

$$\text{Shelf life(days)} = 4.5 \times 10^{-15} \times \exp\left(\frac{9650}{K}\right) \quad (5)$$

With K, storage temperature in Kelvin.

3.6. 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 2. The strongest off-odor producers belonged to the *Enterobacteriaceae* and lactic acid bacteria at 28 °C and 7 °C, whereas at 0 °C the off-odor producing bacteria were *Pseudomonas* spp. and lactic acid bacteria. It should be noted that all the black colonies isolated from iron agar were identified as members of lactic acid bacteria. *Morganella morganii*, a member of *Enterobacteriaceae*, was twice isolated from *Pseudomonas* agar in this study.

4. Discussion

The present study aimed at evaluating the quality change of tropical brackish water shrimp (*P. notialis*). The initial total aerobic counts obtained in this study (5.5 log CFU/g) are higher than those

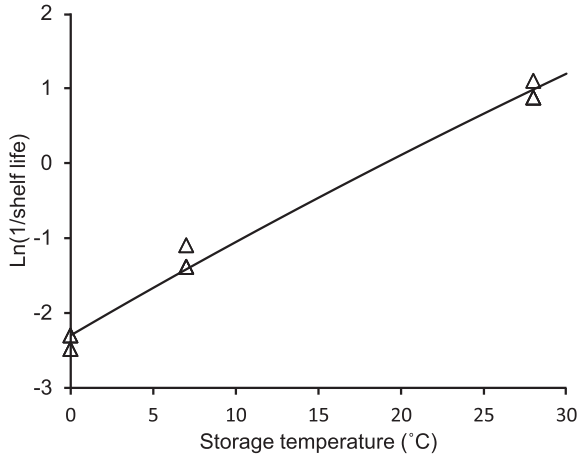

Fig. 4. Shrimp shelf-life modeled with Arrhenius model using data from lake Nokoue and lake Aheme. **Legend**: time is in days.

Table 2
Spoilage potential assessment and identities of isolates capable of producing strong off-odor at 28 °C, 7 °C and 0 °C.

T[a](°C)	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*[e]	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*[e]	KJ626230	70
	PSA[b]	14	1	99% *Morganella morganii*	KJ626253	50
	VRBGA[c]	15	11	99% *Klebsiella pneumoniae*	KJ626250	70
				99% *Klebsiella pneumoniae*	KJ626261	80
				99% *Klebsiella pneumonia*[e]	KJ626227	70
				100% *Klebsiella variicola*[e]	KJ626231	80
				99% *Escherichia fergusonii*	KJ626246	80
				99% *Escherichia fergusonii*	KJ626262	90
				99% *Escherichia fergusonii*	KJ626263	80
				99% *Shigella sonnei*[e]	KJ626232	80
				97% *Proteus vulgaris*	KJ626254	80
				99% *Escherichia fergusonii*	KJ626264	70
				99% *Providencia vermicola*[e]	KJ626236	60
	MRSA[d]	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*[e]	KJ626237	60
	PSA	12	1	99% *Morganella morganii*	KJ626252	60
	VRBGA	11	4	99% *Proteus penneri*[e]	KJ626233	80
				99% *Proteus penneri*[e]	KJ626235	70
				99% *Proteus penneri*[e]	KJ626228	70
				99% *Proteus penneri*[e]	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**			

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

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 7 °C and 28 °C, 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 iron agar 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* (Vogel et al., 2005; Serio et al., 2014), *Shewanella morhuae* (Serio et al., 2014), and *S. 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 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* (7 °C and 0 °C) 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 0 °C (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 15 °C (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 7 °C and 0 °C.

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 0 °C are in agreement with earlier observations that *Pseudomonas* spp. are the dominant microorganisms in tropical shrimps stored in ice (Chinivasagam et al., 1996, 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 *P. 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 *P. psychrophila*.

High storage temperature (28 °C) 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 (7 °C and mainly 28 °C) 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, 2005; Wunderlichova et al., 2013).

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 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 *P. 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 0 °C where TVC counts were lower at sensory rejection time compared to TVC counts at 28 °C and 7 °C. 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 0 °C than the TVC counts at other storage temperatures (4–16 °C). The authors hypothesized that although in low number, the microorganisms had more time (sensory rejection time at 0 °C 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 0 °C 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 (Matches, 1982; Mendes et al., 2002; Lopez-Caballaro et al., 2007).

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 Zeng et al. (2005) (7.41), Lopez-Caballero et al. (2007) (7.3), Mendes et al. (2002) (7.1) and Goswami et al. (2001) (7.2). 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 (100 °C, 30 min) seafood juice (Gram and Huss, 1996; Joffraud et al., 1998; Leroi et al., 1998; Koutsoumanis and Nychas, 1999; Mace et al., 2014). Each of these treatments may fail to reproduce exactly the untreated product due to modification of the physicochemical composition of the product and inactivation of competitive flora (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 the composition. Delfieh et al. (2013) did not find any significant difference in protein and ash contents of raw and boiled (100 °C, 6 min) shrimp. In a comparative study of the spoilage of raw and boiled shrimps stored in 2 °C, 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 preliminary shelf-life model cover temperature variation range in the supply chain (from 0 °C (ice) to ambient temperature (28 °C)). 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 (6) (based on Ratkowsky model) for fresh seafood shelf-life prediction Shelf life(T) = Shelf life$(T_0)/(1 + 0.1 \times T)^2$ (6) (with T, temperature (°C)) (Dalgaard and Huss, 1997) predicts a shelf-life of 16.5 h for a shelf-life of 11 d at 0 °C (the average found in our study) and a storage temperature of 30 °C. This prediction is unrealistic. The equation (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 (7) for tropical fresh seafood shelf-life prediction has been proposed by Dalgaard and Huss (1997). Shelf life(T) = Shelf life$(T_0) \times \exp(-0.12 \times T)$ (7) with T, temperature (°C). This model gives predictions similar to the developed model in this study, especially at high storage temperature. For example, at 30 °C, the generated model in this study (equation (5)) predicts a shelf-life of 7.2 h. At the same temperature (30 °C) and for a shelf-life of 11 d at 0 °C, the model by Dalgaard and Huss (1997) (equation (7)) gives exactly the same prediction of 7.2 h. However, at 3 °C, 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 (0 °C), *Pseudomonas* spp. were dominant, whereas at 7 °C and 28 °C H_2S-producing bacteria were the dominant group of microorganisms. The potential spoilage organisms identified at 0 °C were *Pseudomonas* spp. and LAB. At 7 °C and 28 °C, LAB and *Enterobacteriaceae* were the main potential spoilage organisms. Good correlations were found between the sensory scores and the chemical indicators measured especially with TVBN and 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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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.11.005.

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