

## Quality changes of white shrimp (*Litopenaeus vannamei*) stressed by acute hypoxia and stored under chilled conditions

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### Abstract

The quality changes of *Litopenaeus vannamei* stressed by acute hypoxia and stored under chilled conditions were studied. The stressed group was exposed to hypoxia ( $\sim 0.5$  mg L<sup>-1</sup> DO/5h), whereas the control group to normoxia (5.0 mg L<sup>-1</sup> DO). pH was significantly higher in stressed shrimp and increased throughout storage time. Total Volatile Bases (TVB-N) increased ( $p < 0.05$ ) during storage for both, control (70%) and stressed shrimp (87%). Maximum permissible limits of TVB-N were reached on day 9 and 6 for control and stressed shrimp respectively. In addition, higher Trimethylamine (TMA) concentrations were detected in control shrimp. No pathogens were detected for both shrimp, and the permissible limits of the monitored microorganisms were not exceeded. Ante mortem hypoxia had an effect on pH, but not on the microbiological quality, TVB-N and TMA-N values.

### Keywords

Hypoxia  
Microbiological quality  
Postmortem quality  
*Litopenaeus vannamei*  
Ice storage

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### Introduction

Shrimp produced by aquaculture is the most important crustacean in terms of value, representing 15.4% of the total value of international traded fish products in 2008 (FAO, 2010). In México, shrimp production participates with 37.8% of the total value of national fish products in 2013 (CONAPESCA, 2013). Mexican shrimp production grew from 45,857 tons in 2002 to 100,321 tons in 2012 (CONAPESCA, 2013).

During culture cycle shrimp are exposed to unpredictable and highly variable physicochemical factors (e.g. temperature, pH, salinity, dissolved oxygen, metabolites, and etcetera), which in some cases could be stressors for shrimp (Alpuche *et al.*, 2005, Pillet *et al.*, 2016). Hypoxia is one of the most common causes of stress for farm-raised shrimp; herein, hypoxia events usually occur during night when phytoplankton consumes oxygen, and during harvest process because shrimp increases its breath in a reduced volume of water; in addition, large fluctuations in dissolved oxygen levels are common in estuarine environments inhabited by shrimp, causing considerable economic losses due to the increased mortality, decreased growth rate and the use of anaerobic metabolism to produce energy (causing low protein utilization) in organisms exposed to low

oxygen levels (Ibarra *et al.*, 2007; Brown-Peterson *et al.*, 2008; Jiang *et al.*, 2009; Soñanez-Organis *et al.*, 2012; Pillet *et al.*, 2016).

The response of crustaceans exposed to hypoxia has been well studied from behavioral, physiological, metabolic and molecular perspectives (Brown-Peterson *et al.*, 2008; Jiang *et al.*, 2009; Martinez-Cruz *et al.*, 2012; Jimenez-Gutierrez *et al.*, 2013; Trasviña-Arenas *et al.*, 2013; Pillet *et al.*, 2016); although, there is scarce information regarding how the ante-mortem stressors can affect the post-mortem biochemistry and quality of shrimp, recently Ramirez-Guerra *et al.* (2012) and Garcia-Sifuentes *et al.* (2013) studied the effect of hypoxia on the physicochemical, functional properties and protein stability of white shrimp, concluding that hypoxia had an effect on the physicochemical and functional properties of shrimp stored on ice, but the effect overcame after the frozen process. Other study reported the effect of exercise, emersion and presence of *Hematodinium* sp. on the pH, glycogen, L-lactate and the adenylate energy charge (AEC); the authors concluded that the chronic stressors resulted in a complete ante mortem depletion of abdominal muscle glycogen (Gornik *et al.*, 2010). However, there is scarce information regarding the effect of antemortem hypoxia on the microbiological and postmortem quality of white shrimp. Changes promoted by antemortem hypoxia

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combined with changes occurring during storage (ice and frozen) could have negative implications on the acceptance of shrimp by consumers.

Shrimp is a very perishable product, and postmortem changes may occur rapidly compared to fish (Zeng *et al.*, 2005; Wei-Hsuan *et al.*, 2017). Total viable microbial counts (TVC) and chemical tests, such as analysis of trimethylamine (TMA) and total volatile basic nitrogen (TVB-N), have been used for years in the seafood industry to evaluate spoilage of seafood (Yuan *et al.*, 2016). Limits for these quality indicators are defined in standards, guidelines, and specifications for acceptability.

Considering the above information, the aim of this research was to evaluate the quality changes of the white shrimp (*Litopenaeus vannamei*) stressed by acute hypoxia and stored under chilled conditions.

## Materials and Methods

### Chemical reagents

All chemical reagents used for the parameters studied were reactive grade from Sigma-Aldrich; in addition, agars and culture media for microbiological quality evaluation were also from Sigma-Aldrich.

### Bioassay

Live shrimp were obtained from a commercial farm located at Guaymas, Sonora. Organisms were transported to the laboratory and randomly distributed into two plastic tanks (200 shrimp/tank 2000 L). Specimens at inter-molt stage and weighing approximately 15 g were allowed to acclimate to laboratory conditions for two days. Thereafter, one group (tank 1) was exposed to hypoxia conditions ( $0.5 \text{ mg}\cdot\text{L}^{-1}$  DO) for five hours; during the bioassay, the decrease of DO was monitored until level drop down to  $0.5 \text{ mg}\cdot\text{L}^{-1}$ . The second group (control, tank 2) was maintained under normoxia ( $5.0 \text{ mg}\cdot\text{L}^{-1}$  DO). Water parameters were maintained at the following optimal conditions during the experiment: salinity 3.5‰, pH 6.8-7.0 and temperature  $28 \pm 2^\circ\text{C}$ . Both groups were harvested simulating farm harvesting conditions. Shrimp were immediately placed on coolers within alternate layers of ice-shrimp-ice and transported to the Seafood Laboratory at CIAD in Hermosillo, Sonora, where the cephalothoraxes were removed by hand. The abdominal portions of shrimp at pre-rigor stage (control and stressed) (Díaz-Tenorio *et al.*, 2007) were placed inside plastic bags (eight plastic bags for control and eight for stressed shrimp), then placed on coolers (ice-shrimp-ice) and stored at  $0\text{-}2^\circ\text{C}$  for 21 days. pH, Total Volatiles Bases (TVB-N), Trimethylamine-N (TMA-N) and

microbiological quality were analyzed at days 0, 1, 4, 6, 9, 12, 16 and 21 of storing.

### Muscle homogenate pH

In order to obtain the muscle's pH, a sample of 10 shrimp without shell was homogenized to obtain a fine paste. pH was measured by triplicate at  $2\text{-}4^\circ\text{C}$  using a flexible micro pH electrode (Thermo, Orion 4 star, USA). The pointed tip of the electrode was inserted into the shrimp paste. In order to determine when the pH changes were more evident,  $\Delta\text{pH}$  was calculated.

### Total volatiles bases (TVB-N)

TVB-N was determined according to the procedure of Woyewoda *et al.* (1986). Briefly, the method determines volatile bases by distillation in alkaline condition using magnesium oxide. The TVB-N was evaluated at each during storage at each sampling day (1, 2, 3, 6, 9, 12, 16 and 21 d). Ten-gram sample and 300 mL of distilled water were homogenized into a 1000 mL round bottom distillation flask, using a tissue homogenizer Ultra-Turrax T-25 Basic (IKA® Works, Inc. Wilmington, NC, USA). Two grams of MgO were added and immediately swirled; thereafter, 20 drops of commercial oil were added as antibumping, and the distillation flask was connected to a vertical distillation apparatus. The distillation flask with the sample was heated for 10 min and distillation was carried out for 25 min. The condensate was received in an Erlenmeyer flask containing 2% boric acid solution and titrated back to the original color using a standard solution of  $0.05 \text{ N H}_2\text{SO}_4$ . A blank was titrated using the same reagents without adding the sample. TVB-N was expressed as mg N/100 g sample.

### Trimethylamine-N (TMA-N)

TMA-N was determined by following the picrate method with minor modifications introduced by Woyewoda *et al.* (1986). TMA-N was extracted at each sampling day (1, 2, 3, 6, 9, 12, 16 and 21 days). A 1g sample was homogenized in 6 mL of 7.5% TCA for 2 min using a tissumizer Ultra-Turrax T-25 Basic (IKA® Works, Inc. Wilmington, NC, USA). Thereafter, the homogenate was centrifuged in a refrigerated centrifuge Beckman J2-21 (Beckman Instruments Inc. Palo Alto, CA) at  $3000 \times g$  for 15 min at  $4^\circ\text{C}$  and the supernatant was filtered through Whatman No.1 filter paper. Finally, the extract was frozen at  $-40^\circ\text{C}$  until the appropriate analysis. Results were expressed as milligrams of TMA-N per 100 mg of sample.

Table 1. pH, ΔpH, total volatile bases (TVB-N) and trimethylamine (TMA-N) of control and stressed shrimp stored on ice.

Chill storage time (days)	pH		ΔpH		TVB-N mgN/100g of muscle		TMA-N mgN/100g of muscle	
	Control	Hypoxia	Ctrl	Hyp	Control	Hypoxia	Control	Hypoxia
1	6.62±0.1 <sup>abA</sup>	6.85±0.10 <sup>abB</sup>			23.13±2.85 <sup>a</sup>	22.97±2.11 <sup>a</sup>	1.88±0.6 <sup>aA</sup>	0.73±0.1 <sup>B</sup>
2	6.79±0.23 <sup>ba</sup>	7.04±0.27 <sup>abA</sup>	0.15	0.18	25.60±1.09 <sup>a</sup>	25.60±0.98 <sup>ab</sup>	3.45±0.9 <sup>abA</sup>	2.59±1.7 <sup>A</sup>
4	6.85±0.12 <sup>ba</sup>	7.12±0.23 <sup>bcB</sup>	0.06	0.18	27.21±3.50 <sup>a</sup>	29.60±0.28 <sup>b</sup>	3.80±0.7 <sup>ba</sup>	2.34±1.7 <sup>A</sup>
6	6.98±0.07 <sup>ba</sup>	7.15±0.20 <sup>bcB</sup>	0.10	0.00	31.74±1.06 <sup>b</sup>	33.00±3.13 <sup>b</sup>	3.52±0.8 <sup>ba</sup>	2.13±1.4 <sup>A</sup>
9	7.09±0.19 <sup>cdA</sup>	7.36±0.11 <sup>cb</sup>	0.17	0.21	33.44±2.10 <sup>b</sup>	34.22±1.31 <sup>bc</sup>	4.10±0.7 <sup>bcA</sup>	2.12±1.6 <sup>A</sup>
12	7.27±0.06 <sup>cdA</sup>	7.42±0.15 <sup>cb</sup>	0.11	0.07	34.49±2.35 <sup>b</sup>	37.57±2.0 <sup>c</sup>	2.47±1.7 <sup>abA</sup>	0.90±0.5 <sup>B</sup>
16	7.33±0.05 <sup>cdA</sup>	7.50±0.07 <sup>cb</sup>	0.06	0.1	38.80±3.34 <sup>c</sup>	40.82±1.21 <sup>d</sup>	3.64±0.2 <sup>ba</sup>	2.33±1.5 <sup>A</sup>
21	7.35±0.09 <sup>cA</sup>	7.56±0.06 <sup>cb</sup>	0.07	0.06	39.21±2.92 <sup>c</sup>	42.89±2.43 <sup>d</sup>	3.19±0.5 <sup>abA</sup>	2.38±1.6 <sup>A</sup>

Ctrl: Control, Hyp: Hypoxia. Values are reported as mean ± standard deviation (n=9). Different lowercase letters within the same column indicate significant differences at p<0.05 and different uppercase within the same row and parameter indicate significant differences at p<0.05.

### Microbiological quality

Shrimp without cephalothoraxes (stressed and control) were used for microbiological analysis. A total amount of 10 g of muscle was collected and placed into sterile plastic bags in each sampling day. Aerobic mesophiles, psychrophilic (NOM-092-SSA1-1994) and coliforms (NOM-113-SSA1-1994) were evaluated at each sampling day (1, 2, 3, 6, 9, 12, 16 and 21 days) incubating at 35 ± 2°C and 24 h; whereas *Vibrio cholerae* (BAM. 8va. Ed., 2004, Cap.9), *S. aureus* (NOM-115-SSA1-1994, Baird Parker agar Difco at 35–37°C), *Salmonella* spp (NOM-114-SSA1-1994, Xylose Lysine Desoxycholate XLD agar Difco at 35–37°C) and *Listeria monocytogenes* (NOM-143-SSA1-1995, UVM Modified *Listeria* Enrichment Broth Difco, followed by plating on Oxford MOX, agar Difco, incubated at 30°C/18-24 h) were evaluated exclusively at the beginning and the end of the storage time.

### Statistical analysis

Data were analyzed by performing analysis of variance (ANOVA) using the statistical software package NCSS (version 5.1; Kaysville, UT). Significant differences were detected by performing a Tukey multiple range test, considering a significance level ( $\alpha$ ) of 0.05.

## Results and Discussion

### Bioassay

Dissolved oxygen levels control remained stable with in control tanks, registering an average of 4.53 ± 0.1 mg L<sup>-1</sup> (Table 1). In contrast, a continuous decrease of DO was observed through the first hours (1.75 mg L<sup>-1</sup> and second (1.96 mg L<sup>-1</sup>). Once the level of ~0.5 mg L<sup>-1</sup> (0.46 ± 0.06 mg L<sup>-1</sup>) was registered, it remained constant during the rest of the bioassay

(approximately 5 hours).

### Muscle homogenate pH

pH monitoring has been a common practice in studies of spoilage of fishery products; however, in the opinion of some researchers, sometimes pH may not be an effective indicator of freshness for some species due to their biological variability and harvesting procedures; for this reason, pH must be complemented with other analyses (Howgate, 2009). Usually, pH increases after the resolution of rigor mortis; results of pH exhibited statistical differences (p<0.05) among control and stressed shrimp. In addition, pH level increased (p<0.05) throughout the storage period for both treatments; for instance, pH increased from 6.62 to 7.35 and from 6.85 to 7.56 (Figure 2) for control and stressed shrimp respectively; however, stressed shrimp registered higher values of pH compared to control. Nirmal and Benjakul (2011) reported initial pH of 6.59 in pacific white shrimp stored in refrigeration, and reported pH increments as storage time ran. Okpala *et al.* (2014) described a decrease of 80% of the titratable acidity for white shrimp on 8th day of store in ice. The increase in pH level of shrimp during ice storage was the result of accumulation of basic compounds generated from both, autolytic processes by endogenous enzymes and microbial enzymatic degradations (Nirmal and Benjakul, 2009). Díaz-Tenorio (2006) stored white shrimp in ice and reported that the first significant increase in pH occurred from 3h to 15h; after that (from 15h to 48h) the pH increase was associated to the NH<sup>3+</sup> generated during the synthesis of IMP from AMP. In addition, crustaceans have high intrinsic concentrations of ammonia in muscle as well as enzymes that produce even more.

The highest increase in pH (ΔpH) was found to occur on 12th and 9th days for control and stressed

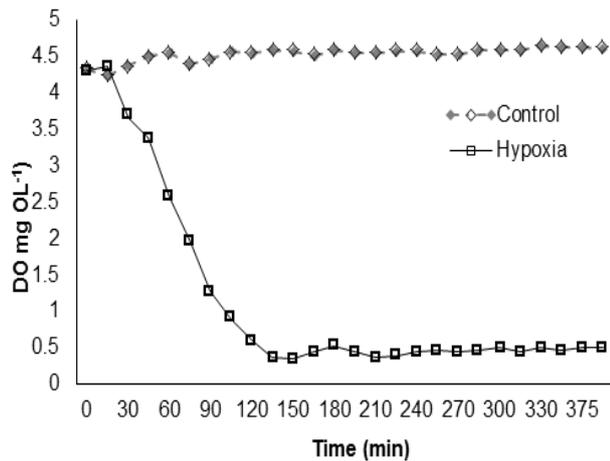


Figure 1. Dissolved oxygen levels registered in water during the bioassay, for shrimp exposed to normoxia (control) and hypoxia.

shrimp respectively (Table 1); results also indicated that the  $\Delta$ pH of stressed shrimp was evident since the beginning of the storage. Changes in pH may represent a promoter of the adequate conditions for microbial growth, considering that mesophiles and psychrophiles increased at 12<sup>th</sup> day of storage as shown in Figures 1 and 2. These data suggests that stressed shrimp could be more susceptible to microbial and endogenous enzymes activity.

#### Total volatile bases (TVB-N)

Total volatile bases and TMA-N are usually considered as quality indicators and are widely used to estimate the freshness of fishery products. These parameters may indicate the occurrence of bacterial spoilages independently of the ageing process, because the autolytic aspects of deterioration are contemplated (Ocano-Higuera *et al.*, 2009). Changes in TVB-N registered in control and stressed shrimp were monitored during ice storage of 21 days (Table 1). At the beginning of the storage (day 1) TVB-N values were  $22.13 \pm 2.7$  and  $22.97 \pm 2.1$  mg N/100 g of sample for control and stressed shrimp respectively; however, maximum permissible limits established by the Mexican Official Standards (35 mg N/100 g sample) were registered at 6<sup>th</sup> and 9<sup>th</sup> days for stressed and control shrimp respectively, suggesting a negative effect of hypoxia on this parameter. Díaz-Tenorio (2006) reported initial a TVB-N value of 21.5 mg N/100 g for recently harvested white shrimp, the study described that the permissible limit was exceeded at day 5, but no ammonium odor was detected. Although the limit of acceptability of TVB-N for shrimp and fish is 35 mg N/100 g, as shown in the present and in another studies, the average of TVB-N for fresh crustaceans sometimes could be higher and in good conditions to consume. In example, Wei Hsuan *et al.* (2017) reported TVB-N

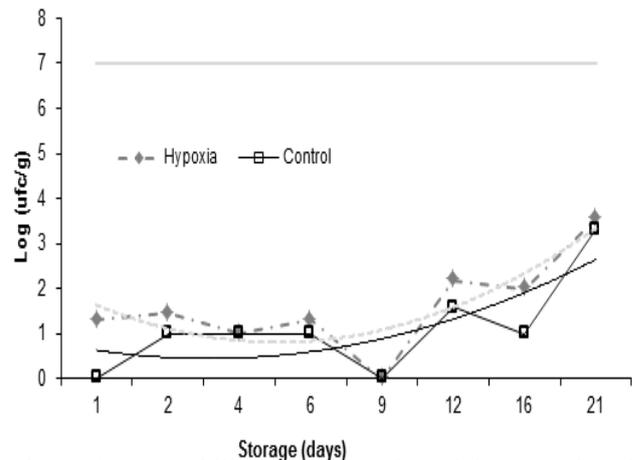


Figure 2. Mesophilic bacteria evaluated in control and stressed shrimp stored on ice.

Curve line represents the trends while breaks line represents the values obtained during the storage. The continuous horizontal line represents the permissible limits of mesophiles.

of 70 mg N/100 g in fresh shrimp under refrigerated storage, while Zeng *et al.* (2005) observed initial TVB-N values of 33.5 mg N/100 g in fresh shrimp under stored in ice.

The results of this experiment also showed that TVB-N increased ( $p < 0.05$ ) during the storage time for both treatments; control increased by 70% while stressed shrimp increased up to 87%. It was reported that the increment of TVB-N level could be caused by different factors; these factors could be proteolysis catalyzed by endogenous proteases, degradation of ATP, some ammonium derived from osmoregulation of the organisms and finally the presence of ammonium previously formed by bacterial deamination of proteins, peptides and aminoacids (Etienne *et al.*, 2005; Díaz-Tenorio, 2006; Howgate, 2010; Yun-Fang, Shen-Ping, Jing *et al.*, 2013). In a recent study, Martínez-Cruz *et al.* (2012) reported a significant increase of 70% in the ATPase activity of the mitochondrial protein extract of white shrimp undergoing hypoxia (after 6h); the authors hypothesized that during the early phase of hypoxia the enzymatic machinery hydrolyzed ATP instead of producing it; when this process occurred, ammonium from autolysis is produced and changes in pH may occur.

The results of this experiment suggest that hypoxia itself may not be a robust factor to have an influence on the post mortem deterioration of shrimp muscle. The adequate post mortem management in this experiment did not allowed hypoxia to have a further negative effect on the muscle's quality; however, it could be hypothesized that hypoxia played a role as a promoter of ATP hydrolysis; this ATP hydrolysis produces ADP, which is further degraded into AMP, with the subsequent IMP conversion and

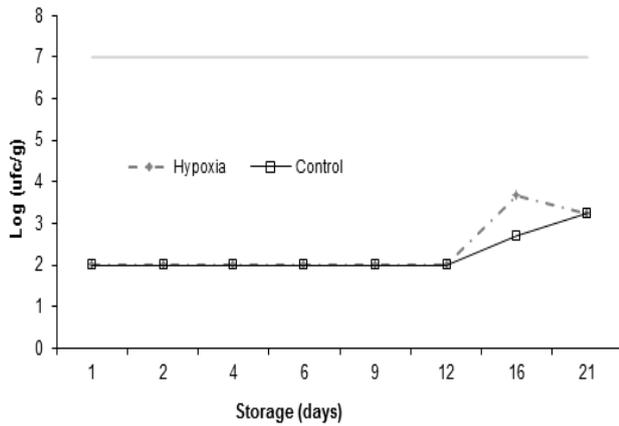


Figure 3. Psychrophilic bacteria evaluated in control and stressed shrimp stored on ice.

Continuous horizontal line represents the permissible limits of psychrophiles.

$\text{NH}_3^+$  production; finally, the ammonium produced is detected in muscle as TVB-N.

From the microbiological perspective, the quality of both control and stressed shrimp were acceptable during the storage period, the permissible limits (Mexican Official Standards and Codex) of the microorganisms studied were not exceeded. On the other hand, other biochemical changes may occur in shrimp muscle in spite of the absence or non-detectable limits of microorganisms; for instance, García-Sifuentes *et al.* (2013) exposed white shrimp specimens to similar hypoxia conditions and reported greater hue angle in stressed shrimp than those exposed to normoxia; greater changes in color occurred after day 9; the study also demonstrated that raw shrimp tend to turn into brown color after the storage in ice. Considering this information and some of the results obtained in this experiment, it could be hypothesized that the true break down of the quality of white shrimp may occur after 9-12 days of ice storing; Zeng *et al.* (2005) and Yun-Fang, Jing, Shen-Ping *et al.* (2013) described a rejection limit of acceptability by sensory scores at day 6.

#### Trimethylamine (TMA-N)

Trimethylamine is formed by the reduction of Trimethylamine oxide (TMAO) by some species in the bacteriological flora of spoiling fish. It is a pungent volatile amine which gives the typical "fishy" odor of spoiled seafood. The changes in TMA-N of control and stressed white shrimp were monitored; at the beginning of the storage TMA-N values were  $1.88 \pm 0.6$  and  $0.73 \pm 0.1$  mgN/100 g of muscle for control and stressed shrimp respectively (Table 1). In contrast to the TVB-N, trimethylamine (TMA-N) value was higher ( $p < 0.05$ ) in control than in stressed shrimp. Control shrimp increased ( $p < 0.05$ ) the TMA-N content during the storage time, whereas

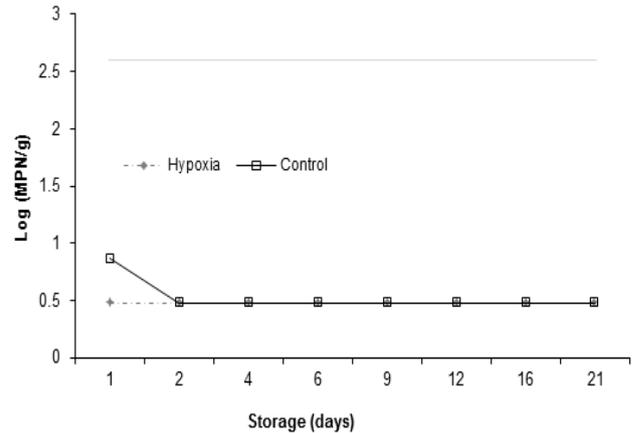


Figure 4. Faecal coliforms (NMP) evaluated in control and stressed shrimp stored on ice.

Results are shown in log MPN/g. The continuous horizontal line represents the permissible limits of psychrophiles.

no differences ( $p > 0.05$ ) were observed in stressed shrimp. Unless no significant differences through the storage time were observed for stressed shrimp, if day 1 is compared vs 21 of storing, TMA-N levels increased ( $p < 0.05$ ) 3.3 times from de initial level. The permissible limit of TMA-N for fresh shrimp and fish has not been considered yet by the Mexican Standards or the Codex Alimentarius. Results of the present experiment showed that the limit of acceptability of 5 mg N/100g was not exceeded for both, control and stressed shrimp during the storage period. However, the highest value of TMA-N was registered at day 9 for control shrimp (4.1 mg N/100g) and at day 2 for stressed shrimp (2.59 mg N/100g). Okpala *et al.* (2014) reported non acceptable TMA-N values for shrimp after 8 days of ice storage ( $5.21 \pm 0.54$  mg N/100g).

In addition, the formation of TMA during spoilage requires that the muscle contains sufficient amount of TMAO for TMA to be formed. Typically, muscle tissue from the marine environment contains adequate amounts of TMAO, and consequently TMA is almost always found in spoiling marine fish (Yancey *et al.*, 2014). Yet acceptable limits were not exceeded in this experiment.

#### Microbiological quality

The shelf life of a product refers to the time that the product can be stored with good physical and sanitary conditions. Shrimp as many food products presents a worthy nutritional quality, texture, and moisture characteristics; thus it is an excellent material that could be used for some bacteria. If bacterial growth is presented, in this case shrimp muscle can reach levels above the limits established by the health authorities, decreasing its quality and subsequently not being safe for the consumer.

Results showed that the microbiological quality of both groups (control and stressed) were acceptable; in addition, the permissible limit of the microorganisms studied were not exceeded. Pathogens such as *Vibrio cholerae*, *Salmonella* and *Listeria monocytogenes* were not detected in any of the samples through the storage on ice.

Results also exhibited no differences for aerobic mesophiles (sometimes referred as aerobic plate count, total viable count or standard plate count) among control and stressed shrimp (Figure 2); however, mesophiles exhibited a slight increase during the storage period. Though the permissible limits were not exceeded during the 21 days of storing, the increment observed during this period suggests that storage periods longer than 21 days or a broken cold chain may cause an increase of mesophiles above the permissible limits. Recent reports showed an initial aerobic mesophiles of 4.45 and 4.19 log CFU/g in pacific white shrimp (Mu *et al.* 2012; Okpala, 2014); these values were reached in this study by the control shrimp at day 21. The microflora of fish and crustacean is composed by the indigenous flora and the microflora of the processing environment. Any handling from the point of harvesting to the table of the consumer has the potential to affect the microflora of the final product (Lyhs 2009). Herein, it was observed that shrimp muscle avoided the proliferation of microorganisms for both treatments.

Regarding psychrophilic microorganisms, no significant differences were detected among control and stressed shrimp (Figure 3); however, similar to mesophiles, psychrophiles increased after day 12 of storage for both control and stressed shrimp. The evaluation of psychrophilic bacteria is essential, considering their relation with shrimp proteolysis, lipolysis, TVB-N and TMA-N content (deteriorative indicators) through prolonged shrimp cold storage; in that period some bacteria could produce metabolites associated to the off-odors and off-flavors as a result of the spoilage (Lyhs, 2009). However, as showed previously, permissible limit of TMA-N was not exceeded through the storage period. Results for fecal coliforms are shown in Figure 4. No differences were detected for fecal coliforms comparing control to stressed shrimp. In addition, no changes were observed through the storage period, coliform bacteria remained stable. The results could be indicative that the microbiological quality was no affected by the hypoxia and that the adequate shrimp post-harvest handling could have buffered any negative effect produced by ante mortem stress.

## Conclusion

Stressed shrimp registered higher values of pH compared to control, but no effect on TVB-N and TMA-N values was observed. The absence of pathogenic microorganisms in shrimp avoided the proliferation of microorganisms. The microbiological quality was no affected by the hypoxia ante mortem, permissible limit of the microorganisms studied were not exceeded. Further studies could investigate the production pathways of ammonium compounds promoted by the hypoxia including amino acid profile.

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