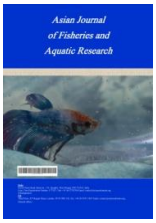

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Benefits of a Biofilm System and Low Ammonia Levels to Decrees the Early Mortality Syndrome in Shrimp Larvae (*Litopenaeus vannamei*) Infected with (*Vibrio parahaemolyticus*)

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

The world shrimp aquaculture, has faced several problems, causing severe losses in shrimp hatcheries; between the most critical has been diseases such as early mortality syndrome (EMS) caused by (*Vibrio parahaemolyticus*). The EMS was initially detected in Asian countries; after, it was disseminated to Mexico and other countries. In Mexico, EMS caused severe economic losses during 2013-2016; and it has not yet been eradicated. Various causes for EMS have been reported; none is entirely accurate, but water quality is essential for successful shrimp aquaculture; therefore, the aim this work was evaluate the ammonia concentration effect on susceptibility to (EMS) on post-larvae (PL-15) shrimp (*Litopenaeus vannamei*) infected with (*V. parahaemolyticus*), using a biofilm system (water with, microalgae, dinoflagellates, protozoa and other planktonic microorganisms). So series of 5 flasks each one were arranged as following: Series S; 900 ml of filtered seawater (FSW) and 10 PL-15 shrimp per flask. Series SB; 840 ml of FSW, 60 ml of biofilm and 10 PL-15 shrimp. Series E; 900 ml of FSW, infected with 2 ml (*V. parahaemolyticus*) 10^6 CFU/ml and 10 PL-15 shrimp. Series EN; fifteen flasks with 900 ml of FSW, 10 PL-15 shrimp, added with NH4Cl (0.535 mg/ml), to get 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l final ammonia concentration in 5 sub-series of 3 flasks each one. During experiment, ammonia concentration and PL-15 shrimp mortality were evaluated in all flask. Ammonia concentration was higher in series EN than in series E; the same was observed in Series S respect to SB, but at lower values. At end of experiment,

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mortality in series EN was 90% Vs 60% in E. Similarly, mortality in series S was 10% Vs 0% in SB. This results confirm that the ammonia increases PL shrimp mortality, and biofilm system reduce ammonia and consequently PL-15 shrimp mortality.

Keywords: Shrimp aquaculture; EMS; Mexico; ammonia; biofilm system.

1. INTRODUCTION

The shrimp aquaculture worldwide has grown vertiginously during last decades. The world production volume of shrimp in hatcheries under controlled conditions has been estimated around of 4.7 million tons in 2018 [1]. In Mexico the aquaculture of white shrimp (*Litopenaeus vannamei*) is the most important cultured specie. As currency income, in 2017 the shrimp aquaculture in the country, had an approximated value of 778 million of US dollars [2], from which 56.3 % was produced in Sinaloa State, located at NW of Mexico, Fig. 1.

Mexico is the seventh largest shrimp producer in the world [1], reaching 227,929 tons in 2017 and Sinaloa was the largest national producer, with 84,426 tons [2]. However, in last decade, diverse diseases have caused severe shrimp mortality, and consequently considerably economic losses for aquaculture farmer. A lot of causes have been reported of these diseases by managers and technicians of shrimp hatcheries; however, accord to personal experience and from reports of many authors, the water quality is one of most important issue in shrimp culture in the hatcheries [3,4,5,6]. On the other hand, microalgae constitute the natural food for shrimp in any aquatic system, and the nutrients amount and its adequate balance, becomes determinant for microalgae growth. Also the physical parameters such as salinity, temperature, total suspended solids (TSS), dissolved gases (CO_2, O_2) are relevant in shrimp culture [3,4]. Concerning to nutrients in water, the N species have a relevant importance, because the nitrite ion (NO_2−) and ammonia (NH_3), are toxic to aquatic organisms. In the aquatic systems, in an oxidation process of two steps, the ion ammonium (NH_4^+) or ammonia (NH_3) are converted to nitrate ion (NO_3^-). In the first step, ammonia is oxidized to nitrite by *Nitrosomas spp.,* bacteria; and in the second step nitrite (NO_2^-) is oxidized to nitrate by *Nitrobacter spp.*, bacteria.

$$NH_3\,(\text{toxic}) + O_2 \rightarrow NO_2^-\,(\text{toxic}) + 3H^+$$

$$NO_2-\,(\text{toxic}) + H_2O \rightarrow NO_3-\,(\text{no toxic}) + 2\,H^+$$

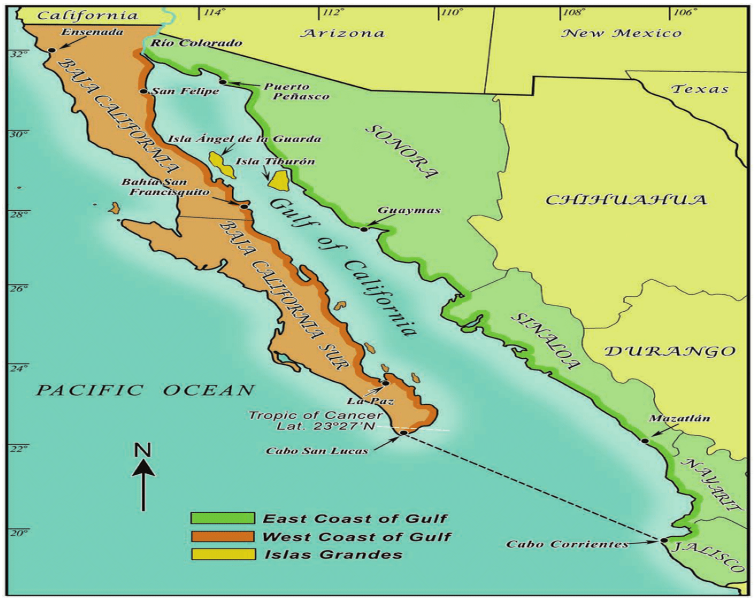

Fig. 1. The Sinaloa State, is located at NW of Mexico. In 2017, the shrimp aquaculture production in the country, had an approximate value of 778 million of US dollars, from which 56.3% was produced in Sinaloa State

In addition to their toxicity, ammonia have been considerate as immune depressing, which make that cultivate organisms like shrimp, becomes more susceptible to diseases, reducing its health condition and then, increasing the shrimp mortality in hatcheries [7,8,9]. On the other hand, in any aquatic reservoir such as shrimp ponds, a biofilm is formed; i.e., a microbial complex formed by bacteria, microalgae, flagellates, ciliates, fungi and other planktonic organism which growth on surfaces and bottom of reservoir. All these microorganisms are being part of the diet of penaeid shrimps. The structure of biofilm depends on substratum features, water fluxes in system, availability of nutrient, light penetration and grazing ability of consuming organism [10]. Some authors claim that an important feature of aquaculture biofilm, is that it can inhibit or compete with pathogenic bacteria into the shrimp pond, and also can reduce the ammonium ion (NH^+_4) concentration by microalgae growth, because they use it as nutrient for its protein synthesis; i.e., the nitrifying bacteria in the biofilm decreased ammonium level in the ponds water [10,11], which is formed when ammonia is dissolved in the water.

$$H_2O+NH_3 \rightleftharpoons OH^- + NH_4^+$$

In fact, the ammonium ion decreases go in parallel to nitrite and nitrate ions increasing in the water; which indicates that nitrifying bacteria in the biofilm play a significant role in the water quality. However, like many other technics or methods for improve the shrimp productivity, is necessary being sure that the implemented technic, enhance the shrimp growth and reducing the incidence of diseases; however, biofilm also may have adverse effects on the shrimp. Perhaps the greatest risk of the biofilm is that pathogenic bacteria, also can growth in the biofilm. Some authors have reported that some pathogenic bacteria including *Vibrio spp.,* can growth on biofilms surface of aquaculture ponds [12,13].

During last years, several authors have reported that bacteria (*Vibrio parahaemolyticus*) has been the cause of the lethal disease known as Early Mortality Syndrome (EMS), also named Acute Hepatopancreatic Necrosis Disease (AHPND) [14]. The infected shrimps by (*Vibrio parahaemolyticus*) show a hepatopancreas with a pale to white color, atrophy and black spot or streaks, and finally a massive mortality of shrimp in few days, in the ponds of shrimp hatcheries [14]. Other authors, report that in last decade, the EMS caused severe economic lost in shrimp hatcheries of Asian countries like China, Viet Nam, Thailand, etc., and after it was disseminated to Mexico and other countries [15]. in Mexico, this disease causes a severe crisis during 2013 to 2016 [1] and it has not yet been eradicated. Therefore, the aim this work was evaluate the effect of ammonia concentration on susceptibility to (EMS) on white shrimp post-larvae (PL-15), infected with (*Vibrio parahaemolyticus*).

2. MATERIALS AND METHODS

In order to know if biofilm reduce the ammonia concentration in the water, and then, the mortality caused by EMS disease in shrimp, or its virulence, a series of experiments was carried out as follows: Groups (series) of 5 flask of 1 L each were arrangement in next way:

Series S. Five 1L flasks with 900 ml of filtered seawater (FSW), passing it by a 5 µm pore size filter, a salinity of 30 Practical Salinity Units (PSU), and 10 PL-15 shrimp in each flask.

Series SB. Five 1L flasks with 840 ml of FSW 30 (PSU), flask, added with 60 ml of biofilm (water from a shrimp hatchery with, green microalgae, cyanobacteria, dinoflagellates, protozoa and other planktonic microorganisms) and 10 PL-15 shrimp in each flask.

Series E. Five 1L flasks with 900 ml of FSW 30 (PSU) and infected with 2 ml of bacteria culture (*Vibrio parahaemolyticus*) 10^6 CFU/ ml and 10 PL-15 shrimp in each flask.

Series EN. fifteen 1L flasks with 900 ml of FSW 30 (PSU), 10 PL-15 shrimp in each flask, added with 1.05, 2.1, 3.15, 4.25 and 5.3 ml of NH_4Cl solution (0.535 mg/ml), in order to get 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l final ammonia concentration in 5 subgroups of 3 flasks each one, and infected with 2 ml. bacteria culture (*Vibrio parahaemolyticus*) 10^6 CFU/ ml.

All flask series were setting out door, to ambient temperature 24-27°C and in a shadow area. Permanent aeration was supplied to flasks using an oil free mini-compressor, plastic pipes 3 mm diameter and porous stones located at flasks bottom, to generate little air bubbles.

To determine the final experimental conditions of series above indicated, several trial and error tests were carried out. Once the conditions of

series were defined, the following analyzes and procedures were performed.

2.1 Ammonia Concentration in Water

To quantify the ammonia concentration in water of series, the indo-phenol method was used [16]. It is based on the absorbance measured at 640 nm wavelength in a spectrophotometer, of the blue color intensity formed by the ammonia reaction with phenol-sodium hypochlorite complex, catalyzed by sodium nitroprusside. The intensity of blue color (absorbance) is proportional to ammonia concentration in the water; i.e., there are a lineal correlation between the absorbance and ammonia concentration. Therefore, every day, 2 ml of water of each flask were taken using plastic syringes, filtered through 0.45 µm pore size filters Pall Life Science®; and then, the following reagents were added:

a) 40 µl of phenol solution and stirring.
b) 40 µl of sodium nitroprusside solution and stirring.
c) 100 µl oxidizing solution (3 ml sodium citrate-sodium hydroxide, 0.8 ml sodium hypochlorite) prepared in fresh and stirring.
d) The samples were kept in dark by 1 hr. All reagent used were supplied by Merck®, Darmstadt, Germany.
d) After this time, the absorbance of samples was read at 640 nm wavelength using a spectrophotometer General Electric Mod. GeneQuant 100®, supplied by Buckinghamshire, UK.

To calculate the ammonia concentration in water samples, an absorbance Vs. concentration correlation was obtained using a sub-standard NH_4Cl solution (0.00535 mg/ml). From this solution, were taken 0.0, 0.2. 0.4, 0.8, 1.2 and distilled water was added to 2.0 ml final volume; then, the reagents as above indicated were added and the absorbance was read. The final concentrations of this sub-standard are show in Table 1.

With this data, the following linear correlation was obtained and the corresponding equation, which was used for quantified ammonia concentration in the water of each flask, during the experiment (Fig. 2).

As can be seen, in the correlation equation, Y=Abs., X= NH_4 conc. (mg/l). Therefore, to calculate the ammonia concentration in water of experiment flasks, the following equation was applied:

$$X= (Y-0.0205)/0.2699$$

2.2 Routine Works in the Series Flasks

The PL shrimp in the flasks were fed two times per day (morning and afternoon) with commercial food (Camaronina®) at rate of 5% total weight. In order to detect abnormal behavior or mortality, the PL shrimp in flask were observed as frequently as it was possible. The shrimps died were removed from the flask immediately, and recorded. Also, feces and feed not consumed were removed from flasks two times per day.

2.3 Infection of PL-15 Shrimp

In order to know if ammonia concentration increased the virulence, and so PL-15 shrimp mortality, the flasks of series E and EN were infected with a culture of (*Vibrio parahaemolyticus*), adding 2 ml (10^6 CFU/ml) in broth culture medium Mueller Hinton Broth (MHB) Difco®, dissolved in 3% NaCl. The mortality was recorded every day at afternoon, until the experiment finished.

Table 1. Absorbance values obtained at (640 nm) of wavelength from 0.0, 0.2. 0.4, 0.8, 1.2 ml of (0.00535 mg/ml) NH_4 sub-standard solution added with distilled water to 2 ml final volume, and NH_4 final concentration expressed in mg/l

ml Std. NH4 0.00535 mg/ml	ml distilled water added	Abs. 640 nm wavelenght	NH4 final conc. (mg/l)
0.0	2.0	0.012	0.0
0.4	1.6	0.308	1.07
0.8	1.2	0.6	2.14
1.2	0.8	0.941	3.21
1.6	0.4	1.135	4.28

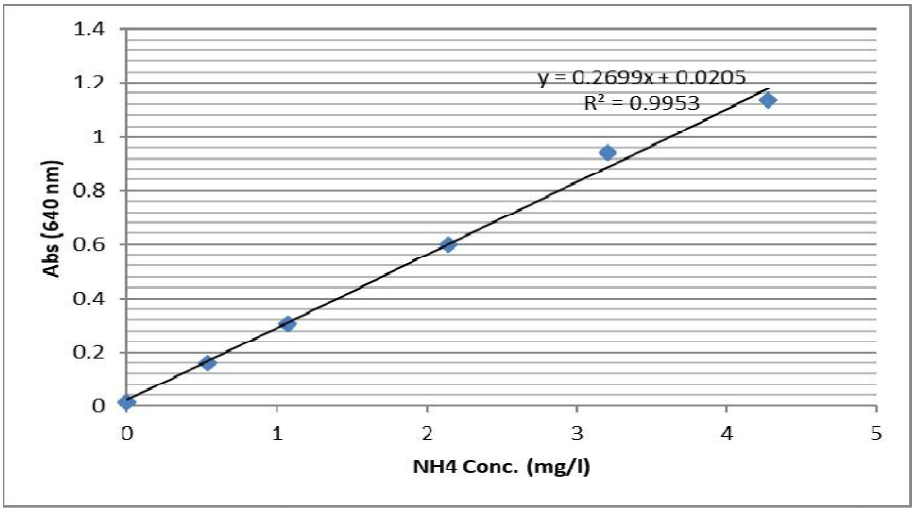

Fig. 2. Linear correlation between ammonium solution NH_4 (0.00535 mg/ml) Vs. absorbance values at 640 nm wavelength, and the corresponding equation

2.4 Statistical Analysis

All data of series experiment were analyzed by (ANOVA) one way, using the Statistica 7.0 software, VinceStatSoftware®. Data which did not meet normality requirements after being transformed were analyzed non-parametrically by Kruskal-Wallis ANOVA and median test.

3. RESULTS AND DISCUSSION

The results of ammonia concentrations in experiment of flask series, are shown in Fig. 3. From these results, the mean and standard deviation were calculated for each series during experimental time (5 days), and then the corresponding graph was made (Fig. 3). As can be observed, the ammonia concentration increased like an exponential way during experimental time; that is consistent with the increase of organic matter in the water. The ammonia and/or ion ammonium, is excreted by aquatic animals and by degradations of particulate and/or dissolved organic matter in aerobic conditions. Also some bacteria can degrade organic matter to ammonia in anaerobic environments, such as ponds bottoms. The ion ammonium can be converted to ammonia and vise verse, depending of water pH; so at pH over 9 the ammonia increase and ammonium decrease, whereas at pH lower than 7.5-8 the ammonium ion increase and ammonia decrease. This is a system in equilibrium depending of water pH; such as is presented in the chemical reaction above indicated.

As can see, the ammonia concentrations of series SB were significant lower than in series S. The same can be observed in series E, which were significant lower than series EN, except in third day, but at higher values. This means that biofilm consumed the ammonia from organic matter produced by PL-15 shrimp feces and uneaten food, and after degraded by bacteria.

On the other hand, the shrimp mortality was higher in series EN than in the series E (Fig. 4). Again, it may be due to ammonia concentration, because in both series, the PL-15 shrimp were infected at same time and with same amount of bacteria (*Vibrio parahaemolytics*); the same can be observed between series S and series SB, but the mortality was ≤ to 10%.

This result becomes to confirm the hypotheses of this work, i.e., the biofilm reduces the ammonia concentration in water of PL-15 shrimp culture; and high ammonia values increase the PL-15 shrimp mortality. Therefore, although these results are preliminary, they are promising, because one of the most severe problems in shrimp hatcheries is the EMS disease, which can produce until 100% of mortality in few days, and there is not an easy way to stop the disease dissemination. On the other hand, high ammonia concentration is a very common problem in shrimp aquaculture. It is produced by excess of organic matter; particularly in intensive and super intensive culture systems, because these culture systems require high amount of feed for satisfy the food demand by shrimps. Also, as

consequence of elevate ammonia values, the immune system of shrimps may be depressed; and consequently, shrimp becomes more susceptible to diseases. This is a vicious circle, because it is common that hatchery managers want to obtain larger shrimp harvest, increasing the shrimps amount per m^3, which require to increase the feed amount, consequently an increase of ammonia. This practicum has produced a lot of economic losses in all shrimp farms of many countries; therefore, an economic and easy way to solve this problem would be the use of biofilm in shrimp farms. However, other authors claim that it is possible that a diversity of *(Vibrio Parahaemolyticus*) varieties or strains, could be the cause of EMS disease in shrimp culture ponds; but all varieties are causing elevate mortality in few days [17]. Several authors have reported that a biofilm with a good complex of microorganisms, enhance the immune response of cultivate shrimp, increasing the haemocytes amount in shrimp haemolymph and the activity of phenol-oxidase enzyme; the same, authors, claim that biofilm also can improve the shrimp growth since them consuming the suspended particles and consequently, improving the water quality [18,19]. Other authors have reported that shrimp larvae are opportunistic feeders; i.e., if diatoms are the dominant specie in the pond, they will prefer this species as diet until be almost or totally consumed; consequently. Also, the composition and diversity of microalgae becomes to determine the nutritional quality of natural food available for shrimp larvae; on the other hand, diatoms have a benefic effect on growth and survival of shrimp larvae, because they are rich in polyunsaturated fatty acids, which can improve growth and the metamorphosis in shrimp larvae, and so, increase the survival and resistance to stressing conditions [20,21]. Also, some authors have reported that extracts of microalga (Dunaliella spp.), can increase the resistance to disease known as white spot syndrome virus WSSV and then, can enhance the growth of cultured shrimp [22]. In fact, biofilm system, is just the way as the ecosystems work, i.e., in the natural environment, always a lot of micro and macro organisms co-habiting the system, then, an equilibrium is reached; therefore, the possibility of some pathogen or predator growth uncontrolled, will be lower than in culture ponds.

There is other system very close to biofilm, named biofloc. The biofloc is a conglomerate of microalgae, bacteria, ciliates, dinoflagellates, foraminifera, and particulate organic matter such as dead organisms, and feed no consumed. The biofloc is bonded together by bacterial secretions, and/or by filamentous microorganisms, and also by electrostatic forces [23]. This system is used in places where water

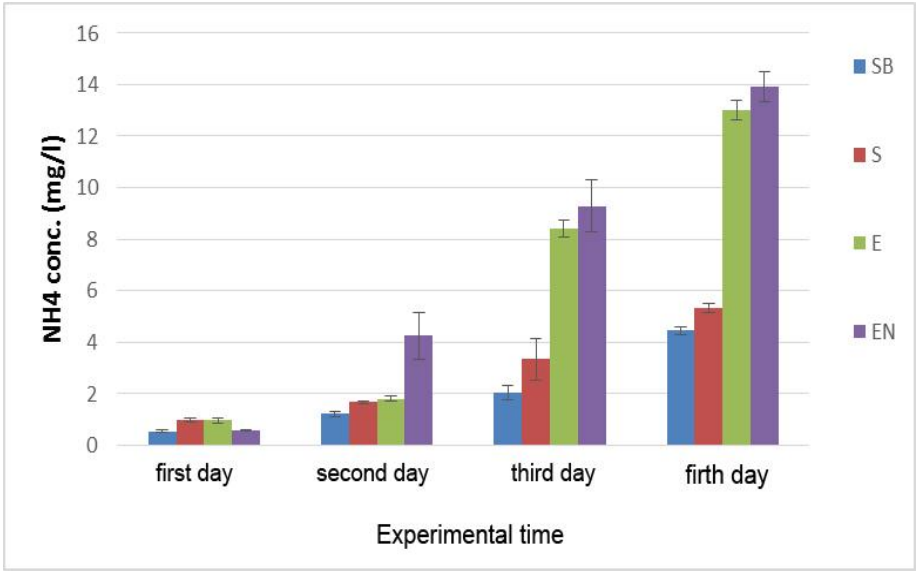

Fig. 3 Ammonia concentration in water from series SB, S, E and EN.
The values of series SB were significant lower than series S. Also the concentration in series E, were significant lower than in series EN ($p \leq 0.05$), except in third day

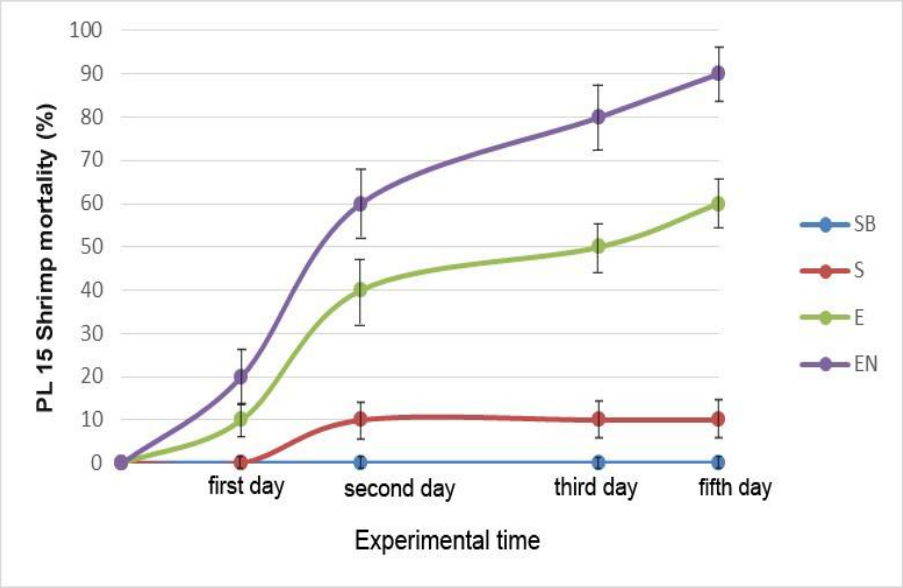

Fig. 4. The PL-15 shrimp mortality in series EN was significant higher than series E ($p \leq 0.05$), although both were infected at same time and with same amount (10^6CFU/ml) of (*Vibrio parahaemolyticus*). Also, mortality is series S was higher than series SB, but a values ≤ to 10%

is not abundant or in farms where intensive aquaculture is practiced to increase shrimp production and/or in estuarine areas where many shrimp farms performing water exchange; then, disease would spread among farms; therefore, reducing water exchange is a good practicum for improving farm bio-security. Other authors reported that in a biofloc system, the weight increase of PL shrimp at 48 days were 670 mg, compared to 640 mg, with a clear water system and 590 mg, with a hybrid system; also in the biofloc system, 13% of the C and 34% of the N in shrimp tissue could be produced from biofloc compounds [24]. Other author claim that in last decade, the biofloc system, has emerged as a new technology for sustainable aquaculture, which may contribute to food security stablished by FAO sustainable development goals [25].

However, in order that the biofloc system work, an extra source of carbon and nitrogen must be supplied. Normally, the carbon source is molasses, and a height protein feed (38%) for nitrogen; on the other hand, to avoid that the flocs dropping down to pond bottoms, an intense aeration is required, therefore an increase of energy supplied to ponds of hatcheries; consequently, the biofloc system in more expensive than biofilm system. Also, biofloc system require that a rigorous control of bacteria be performed; other way, several pathogenic bacteria can growth in the system, which may become a risk for a healthy shrimp growth. Some authors claim that principal problem in biofloc system, is controlling the composition of bacterial community for obtain an adequate water quality, and so to get optimal shrimp healthy conditions; however, the same authors report that (*Vibrio spp*.) was at lower amount in biofloc system comparing with a clear water system [26].

In order to solve some of problems above indicated, the managers and technicians of shrimp farms have used a lot of substances and manipulations in the culture ponds, but the results have been un-satisfactory; moreover, it has resulted in a decrease of water quality due to substances added (antibiotics, alkalis, flocculants, probiotics, etc.) which have increasing the water pollution, since many times it is drained to the environment without any treatment. For instance, in Mexico, a recent official declaration, said that in the Sinaloa coastal systems, the shrimp catches have plummeted to a daily average of about 11.2 kilograms per boat, and between 2015 and 2016 were recorded the lowest catches in the history [2]. However, the fishermen say this could be due to shrimp farm wastes pumped to coastal ecosystems during the larvae growth period, which may be impacting the mortality of shrimp larvae.

4. CONCLUSION

From the results obtained, it can be concluded that ammonia concentration in the water where shrimp are growth, becomes decisive to avoid the development of diseases such as EMS; i.e., the best way to keep the culture ponds in biosafety conditions, is maintaining permanently a biofilm, which will help to shrimp to growth in better health conditions, avoiding that diseases have less chance to infect the shrimps; therefore, its immune system will be performing more efficiently. On the other hand, the biofilm will reduce the amount of food supplied to culture ponds, since it is an excellent natural food for shrimp. In other words, the success of shrimp aquaculture is simply carrying out the farming process as similar as possible to how it occurs in the natural ecosystem; this is concordant with a 30% of mortality reduction in series E (added with biofilm) Vs series EN, although both were infected with same amount of *(V. parahaemolyticus)* bacteria. All this is concordant with comments and recommendations given by other authors [27], e.g., between most important factors to EMS develop, are including: the use of post-larvae infected with *(V. parahaemolyticus)*, intensive systems which require excessive feeding, elevate level of H_2S, due to putrefaction of organic matter, excessive use of calcium oxide to disinfect the ponds, insufficient aeration, use of biofloc system, not use of water with salinity inferior to 20 psu, use of saponin in preparation steps, and use of probiotics and prebiotic.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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