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Effect of ammonia stress on immune variables of Pacific white shrimp *Penaeus vannamei* under varying levels of pH and susceptibility to white spot syndrome virus

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ABSTRACT

Of late, Pacific white shrimp *Penaeus vannamei* culture has intensified globally and is a major contributor to the cultured shrimp produced worldwide. Intensification of its culture has led to elevated ammonia concentration during grow-out. Ammonia toxicity is a function of water pH, temperature, salinity and beyond the optimum range, creates stress to cultured aquatic species which can reduce growth, increase susceptibility to diseases and eventually mortality. The present study was aimed at quantifying the toxic effect of total ammonia nitrogen (TAN) (1, 3, 6 & 9 mg/l) and pH levels (6, 8 & 10) individually and in combination on median survival (50% lethal time) of shrimp (8 g) after exposure for 14 days followed by post-stress challenge with white spot syndrome virus (WSSV) for 9 days. Mortality risk factor and the toxicity effect on the immune variables were evaluated. Individual stressors showed a risk factor of 1–13 times, whereas combined treatments considerably increased the risk of dying compared to control. Low survival (15%) was observed in pH6TAN9 and pH10TAN3 treatments and was substantiated by prominent histological obliteration in gills of shrimp. The cumulative mortality in post-stress WSSV challenged trials was 1–5 times and 1–35 times in individual and combination treatments, respectively compared to control. The study revealed that variations in ammonia and pH beyond the optimal range significantly influence the non-specific immune mechanisms in *P.vannamei* and increases the susceptibility to WSSV especially in combination treatments.

1. Introduction

Intensification of Pacific White Shrimp *Penaeus vannamei* culture affects pond water quality and ultimately shrimp production. The build-up of toxic nitrogenous waste is an important limiting factor in intensive culture systems. Ammonia is the common toxicant resulting from faecal matter and organic detritus arising from the decomposition of organic matter by microorganisms in the water (Chen and Kou, 1996). In aquatic environment, total ammonia nitrogen (TAN) exists both as unionized ammonia (NH_3) and ionized ammonia (NH_4^+) forms (Armstrong et al., 1978). The relative proportions of NH_3 and NH_4^+ depends on pH, temperature and to a lesser extent salinity (Randall and Tsui, 2002), climate change induced parameters. Occasionally, culture ponds contain more than 2–3 mg/l of TAN (Boyd and Musig, 1992). However, in intensive ponds at later stages of culture, TAN concentration can reach as high as 6.5 mg/l (0.15 mg/l NH_3-N) to 46.1 mg/l (0.87 mg/l NH_3-N) (Chen and Liu, 1988; Chen et al., 1989). The unionized form of ammonia is extremely toxic to shrimp (Chen and Lei, 1990) due to its ability to diffuse across cell membranes (Emerson et al., 2011; Fromm and Gillette, 1968).

Changes in water pH can be attributed to many biological and chemical reactions occurring in the pond. In pond water, pH levels fluctuate from 6.6 to 10.2 due to removal of carbon dioxide during day time for photosynthesis of plants and the release of carbon dioxide during the night by both plants and animals (Boyd, 1990). During heavy rain, erosion of acidic soils from dikes into ponds decreases water pH to as low as 4.1 (Boyd, 1989; Chen and Chen, 2003; Haines, 2004). The upsurge in soluble organic matter concentration may result in the formation of red tide that affects pond water quality, consequent to which the pH increases to 9.0 (Wang et al., 2002). Several authors have reported that variations in pH have a significant impact on the physiology of shrimp (Cheng and Chen, 2000; Li and Chen, 2008; Wang et al., 2002; Zhou et al., 2009) and can affect survival (Vijayan and Diwan, 1995; Wang et al., 2002), growth reduction (Allan and Maguire,

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1992) and mass mortalities (Chen and Chen, 2003; Distefano et al., 2008; Li and Chen, 2008; Wang et al., 2002).

In general, crustaceans have the ability of osmotic physiological adaptations to changes in external environment by hemolymph osmoregulation and ion-transport enzymes in gill membrane (Morris, 2001). The gills are a multifunctional organ that provide the selective interface between the external and internal environment and are responsible for homeostasis (Freire et al., 2008; Lucu and Towle, 2003). Nitrogenous compounds can injure the gill tissue of aquatic animals, affecting the oxygen transport, causing mortality (Barbieri et al., 2016; Campos et al., 2015; Lin and Chen, 2003). Changes in pH also have a significant role in gills mutilation because of direct action of H^+ or OH^- ions on gill membranes (Ferguson, 1988; Playle and Wood, 1989). Aquaculture impacted by complex blends of various environmental dynamics including chemical toxicants and pond water variations are not necessarily independent of each other. Farmed shrimp may encounter the combination of several factors simultaneously in a pond, which may be stressful and could affect the physiological responses (Zhou et al., 2009). As a consequence of various adverse environmental parameters resulting in impairment of the host defense system (Perazzolo and Barracco, 1997), decrease in immune response such as total hemocyte count (THC), activity of prophenol oxidase (PO) and superoxide dismutase (SOD) have been reported (Le Moullac and Haffner, 2000; Rodríguez and Le Moullac, 2000). Therefore, the physiological response of shrimp to these environmental stress parameters is of primary concern for shrimp farmers.

Diseases are the primary constraint in aquaculture and white spot disease (WSD) is the most contagious viral infection considered as a major havoc which causes 80–100% shrimp mortality within 3–7 days (Lightner, 1996; Vlak et al., 1999) and consequent economic loss (Primavera, 1997). Till date, no treatment is available and prophylactic measures have been considered as the only means to prevent viral outbreaks (Kautsky et al., 2000; Sánchez-Martínez et al., 2007). Many findings on the role of single environmental stressor causing WSD focused on stress and documented the interrelation of white spot syndrome virus (WSSV) outbreaks and ecological factors (Chen and He, 2019; Fouzi et al., 2012; Liu et al., 2006; Rahman et al., 2006; Ruiz-Velazco et al., 2010; Tendencia and Verreth, 2011).

Several studies have been carried out to determine the toxicity levels of ammonia and pH in different life stages of penaeid shrimp, *Penaeus monodon*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis* and *Peneaus vannamei* (Chen and Lei, 1990; Chen and Lin, 1991, 1992; Cheng et al., 2004; Chin and Chen, 1987; De Lourdes Cobo et al., 2014; Gunalan et al., 2010; Li and Chen, 2008; Lin and Chen, 2001; Magallón Barajas et al., 2006; Pan et al., 2007; Xue et al., 2017). These studies elucidated the effects of environmental factors on WSSV outbreaks and shrimp mortality. Most of the earlier reports were related to individual stressors, but shrimp health, as we know is continuously influenced by a combination of environment stressors, information on which is sparse. Earlier studies have focused primarily on the effect of only one environmental factor at a time on WSSV outbreaks while ignoring the roles of other important factors in pond environment that also determine rates of WSSV replication and shrimp mortality. Hence it was felt necessary to study the effect of two crucial water quality parameters, pH and TAN individually as also in combination on the survival and immunological functions of *P. vannamei* and susceptibility to WSSV infection. To the best of our knowledge this happens to be the first report on the effect of combined stressors on the susceptibility of shrimps to WSSV. The results from this study would bring out important information on the effect of a combination of stressors on shrimp thereby aiding in formulation of safety measures to be taken by aquaculture farmers to maintain the a stress-free as also disease free pond environment.

2. Materials and methods

2.1. Acclimatization of shrimp

P.vannamei (ABW: 8.0 g) were collected from a farm located at Kattur Village, Thiruvallur District of Tamil Nadu and transported with proper aeration to the wet lab facility at Central Institute of Brackishwater Aquaculture (CIBA). Before initiation of environmental stressors toxicity study, the animals were acclimatized in 5000 l FRP tanks for 10 day at laboratory conditions in filtered seawater (salinity: 20 ppt; temperature: 29.3 °C; pH: 7.8 and dissolved oxygen 7.2 mg/l). Shrimps were fed pelleted feed (35% crude protein) @ 3% of the body weight twice daily. Uneaten feed and faecal matter were removed daily by siphoning. Water exchange was carried out once every two days.

2.2. Test solutions for environmental stress experiment

To achieve the desired acidic and alkaline pH levels, by gradual addition of 1 mol/l HCl pH was reduced to 6.0 and raised to 8.0 and 10.0 with 1 mol/l of NaOH (Pan et al., 2007). A stock solution of ammonia-N of 1000 mg/l was prepared with ammonium chloride (Lin and Chen, 2001). The stock solution was diluted to obtain the desired concentrations of 1, 3, 6 and 9 mg/l of TAN. The actual concentration of TAN in test solution was measured using the standard APHA method (American Public Health Association, 1998) and unionized ammonia (NH_3) value was calculated based on the temperature, salinity, and pH of water in each tank by the Russell formula (Erickson, 1985).

2.3. Preparation of WSSV inoculum

The WSSV was extracted from the tissues of severely infected farmed *P. vannamei* collected earlier and confirmed to be positive for WSSV by nested PCR (De La Peña et al., 2007) using primers designed by Kimura et al. (1996). Briefly, the PCR amplification was carried out in a 25 µL Ampliqon III, 2x master mix RED reaction mixture (0.4 mM dNTPs, 0.2 units/µL Ampliqon Taq DNA polymerase, Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20®, Inert red dye and stabilizer, Ampliqon A/S, Denmark), 100 ng of template DNA along with positive and negative controls with the primers sets P1: 5′-ATC ATG GCT GCT TCA CAG AC 3′ and P2: 5′-GGC TGG AGA GGA CAA GAC AT 3′, in the first step PCR in a thermocycler (Effendorf, CA). The cycle parameters were as follows: initial denaturation at 95 °C for 5 min and 30 cycles of denaturation (95 °C for 60 s), annealing (57 °C for 60 s), extension (72 °C for 60 s) followed by a cycle of final extension at 72 °C for 5 min in first step PCR amplification. The nested step was carried out with the same programme by adding one µl of the first step PCR amplification product as a template, using the primers P3: 5′-TCT TCA TCA GAT GCT ACT GC 3′ and P4: 5′-TAA CGC TAT CCA GTA TCA CG 3′. The expected PCR amplicons in first and nested PCR were 982 bp and 570 bp respectively. After amplification, 5 µL of the PCR products were resolved on an agarose gel in Tris-acetate-EDTA (TAE) with 0.5 µg/ml ethidium bromide along a 100bp DNA ladder (Gene Ruler™ 100bp DNA Ladder, Fermentas, Germany) for 30 min (120 V and 30 mA). The amplified PCR products were visualized under UV light, and images documented using a gel documentation system (Bio-Rad, USA). The samples found WSSV positive were stored at −80 °C. The WSSV inoculum was prepared as described by Jiang et al. (2006). After removal of the exoskeleton, the gills and pleopods tissue (0.5 g) from the infected shrimp were homogenized and pooled in 5 ml phosphate buffered saline (PBS) (0.01 mol/l, pH 7.4). The homogenate was centrifuged at 1000 × *g* for 15 min at 4 °C. The supernatant was filtered through a 0.2 µm membrane filter. The filtrate was used as the source of fresh viral inoculum to infect the experimental shrimp.

Apparently healthy *P.vannamei* shrimp (N = 30; ABW: 16) obtained from a nearby shrimp farm, were maintained in 100 l tanks (10 shrimp per tank, at room temperature: 27 °C and salinity: 20 ppt) in wet lab for

three days. The animals were injected with 100 µl WSSV inoculum intramuscularly into the base of the fourth abdominal segment and maintained for seven days. After three days post-infection, pleopods tissue from the WSSV infected shrimp were screened for WSSV by PCR and upon confirmation, the shrimps were stored at −80 °C for further use. The infected tissues of these animals were used for the oral challenge studies.

2.4. Experimental design of environmental stress trials

Experiments were conducted in two clusters, A and B in 1000 L FRP tanks containing filtered seawater stocked with shrimp (N = 80) in each tank. During stress experiment, shrimps were exposed to individual environmental stressors of pH (6, 8 and 10) and TAN (1, 3, 6 & 9 mg/l), and their combination in triplicate for 14 days in both the clusters comprising 7 individual and 12 combination treatments, and control (pH - 7.7; TAN - 0.17 mg/l). The shrimps from the tanks in Cluster A were randomly sampled at weekly intervals for immunological assay. Cluster B treatments were used exclusively for recording survival data. After 14 days' exposure of shrimp to pH and TAN stress, the remaining shrimp in all the treatments from both clusters A and B were pooled under respective treatments in 500 l FRP tanks (N = 20 in each) in triplicate. The shrimp were starved for a day and thereafter challenged orally with WSSV.

2.5. Experimental design of WSSV challenge trials of post stressed shrimp

After two weeks of post-stress experiment, all the treatments were challenged with WSSV except pH10TAN6 and pH10TAN9. Shrimp in all the tanks were starved for 24 h prior to infection with WSSV to eliminate variations caused by the food consumed (Hall and Van Ham, 1998). The next day, all the treated groups were fed with minced WSSV infected shrimp tissue (5% of body weight) twice daily for two days (Di Leonardo et al., 2005). Thereafter, the animals were fed commercial pellet feed. In the control, the shrimp were fed with minced tissue of healthy shrimp followed by commercial pellet feed. Survival data of post stressed WSSV challenge shrimp was recorded in all the tanks. The moribund and dead animals were removed from the experimental tanks and stored at −80 °C for WSSV confirmation.

2.6. Analysis of water parameters

Temperature, pH (Digisun digital pH meter), dissolved oxygen (Fisher Scientific DO meter), salinity (Atago Refractometer), TAN and nitrite-N (Shimadzu UV-1700 Spectrophotometer) were measured daily following standard APHA methods (American Public Health Association, 1998). Temperature (29.3 °C), salinity (20 ppt) and DO (7.2 mg/l) were maintained in all the tanks. Water samples were collected in all the experimental tanks every day, estimated pH and TAN and the minor variations were adjusted with test solutions.

2.7. Molecular diagnosis of WSSV

2.7.1. DNA extraction

Total DNA was extracted from gills and pleopods tissue (50–100 mg) of WSSV-infected shrimp using DNAzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.7.2. WSSV detection by PCR

WSSV detection in post stress WSSV challenged animal tissues was accomplished by using PCR method as described in section 2.3.

2.8. Hemolymph collection and sample preparation

The hemolymph sample (500 µL) was withdrawn from the ventral sinus of each shrimp into pre-chilled 1.0 ml disposable sterile syringes (25-gauge). From this 250 µL, hemolymph was added into the micro centrifuge tube containing 0.25 ml of anticoagulant solution (27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5), mixed well and used for further analysis.

2.9. Assays of immune variables

2.9.1. Total hemocyte count (THC)

For determining the THC (Cells/ml hemolymph), 25 µL aliquots of 1: 1 (V/V) hemolymph and anticoagulant were diluted further to a final 1: 4 dilution with 4% formalin and 0.45 M NaCl, thoroughly mixed and placed in a hemocytometer (Naeubauer chamber) for counting (Bautista-Covarrubias et al., 2014) using a phase contrast microscope (Leica DMIL, Germany).

2.9.2. Phenoloxidase (PO) activity assay

The haemocyte lysate was prepared as follows: to the haemocytes collected in the anticoagulant (200 µL) ice-cold sodium cacodylate buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 7.0) was added thereafter homogenized using sonicator for 20s, centrifuged at 15,000 × *g* for 10 min at 4 °C and the resulting hemocyte lysate supernatant (HLS) was used immediately to measure phenol oxidase activity. Phenol oxidase activity was determined by measuring L-dihydroxyphenyl alanine (L-DOPA) using the methodology of Perazzolo and Barracco (1997). Briefly, 50 µL of HLS was incubated with 50 µL of elicitor (0.1% trypsin in Cacodylate buffer) at 25 °C for 10 min. About 50 µL of the enzymatic substrate L-DOPA (3 mg/ml) was added and incubated for 5 min at 20 °C. The reaction was stopped by addition of 850 µL of distilled water. An increase in the absorbance due to the formation of dopachrome was immediately read at 492 nm using a UV Spectrophotometer (Shimadzu UV-1700).

2.9.3. Superoxide dismutase assay

The hemolymph SOD activity was determined following the method of Richard et al. (2015). Anticoagulant-free hemolymph (20 µL) was placed in a 1.5 ml sterile Eppondorf tube and diluted with 480 µL phosphate buffered saline (PBS). It was then homogenized on ice with a sonicator for 1 min and centrifuged at 17,345 × *g* for 5 min. The SOD activity was measured by reduction of water-soluble formazan dye, and assayed using the SOD assay kit (Sigma, 19160) following the manufacturer's instructions. Briefly, 20 µL of the sample solution and 200 µL of Water Soluble Tetrazolium salt (WST-1) working solution were added and gently mixed. The reaction was initiated by adding 20 µL of xanthine oxidase (XO) and xanthine mix (enzyme solution), which form the superoxide anion. Two blanks were performed by replacing the sample solution with milliQ-water (background reduction of O_2). After incubation at 25 °C for 20 min, the reduction of WST-1 by O_2^- produces a yellowish formazan dye, the absorbance of which was read at 450 nm with UV-Spectrophotometer (Shimadzu UV-1700).

2.10. Gills histopathology

For routine histopathology, the standard procedure of Lightner and Bell (1998) was followed. Sections of 4–5 µm thick were cut using an RM2125RTS rotary microtome (Leica, Germany), stained with Haematoxylin and Eosin (H&E) and mounted with DPX (Sigma, MO, USA). Observations were recorded, and digital images captured using a microscope attached with a digital camera (Leica DMIL, Germany).

2.11. Statistical analysis and data evaluation

The significance of the effect of stressors (pH, TAN) and their combination on the immune parameters (PO, SOD, THC) at different sampling time was tested using ANOVA. The hazard of stressors on shrimp survival after the experiments exposed to stressors and post stress WSSV challenge was analyzed in a Cox proportional hazard

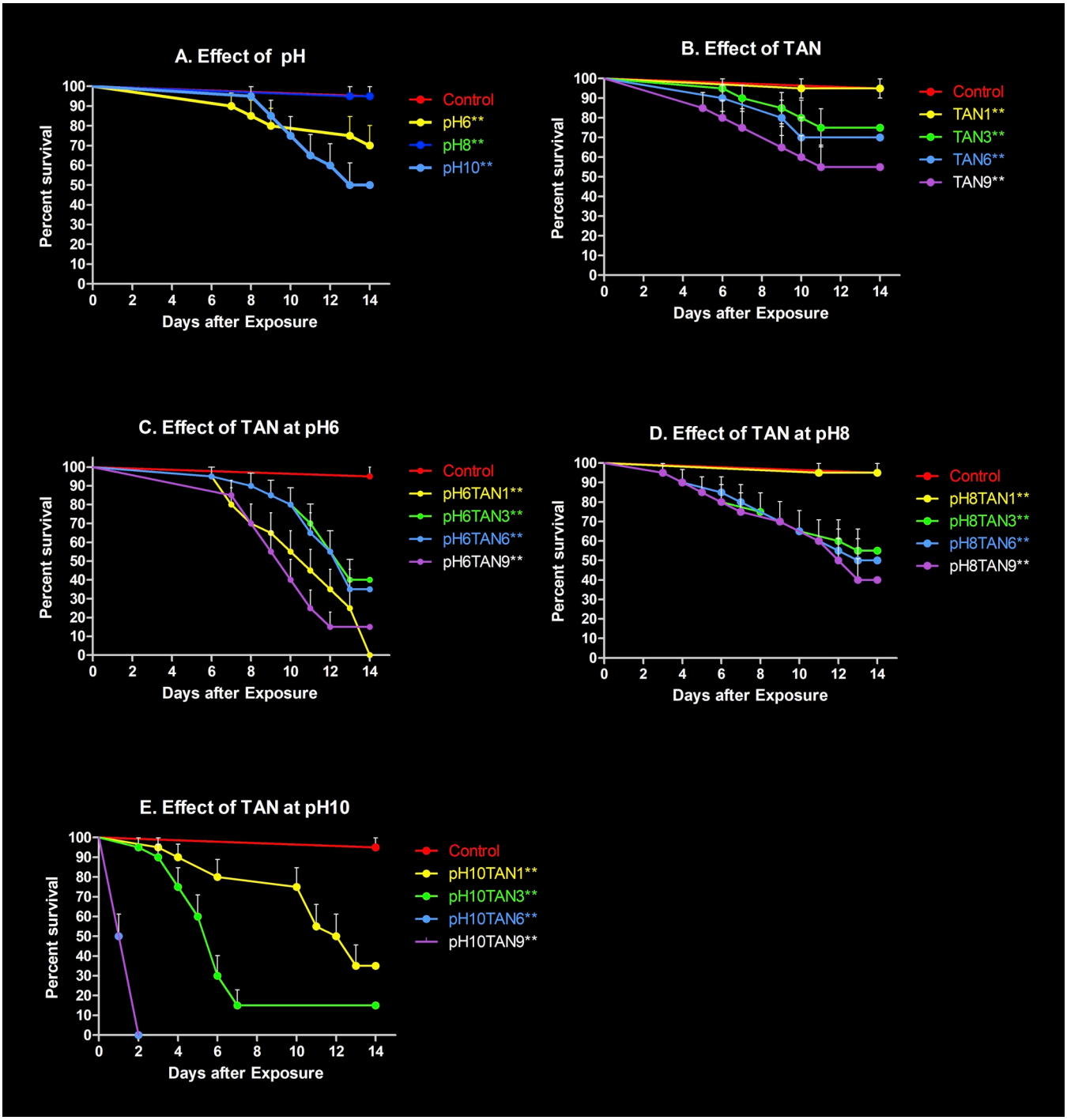

Fig. 1. Shrimp survival proportions (%) (Mean ± SE) of individual pH and TAN and combined treatments (pH X TAN): (A) Effect of pH (B) Effect of TAN (C) Toxic effect of TAN at pH6 (D) Toxic effect of TAN at pH8 (E) Toxic effect of TAN at pH10. ** indicates P ≤ 0.01.

model (Bewick et al., 2004). The relationship between unionized ammonia and other water parameters was computed using the Pearson's correlation coefficient. All the analyses were done using SPSS version 21 software (Armonk, NY, USA). The Kaplan–Meier survival curves were plotted in Graph Pad (Version 7.04 for Windows, San Diego California, USA) and tested for significance in survival with Log rank (Mantel-Cox) test and Log rank test for trend. The statistical significance was tested for all the parameters at p ≤ 0.05 and 0.01 and denoted as * and ** respectively.

3. Results

3.1. Effect of pH and TAN stress on survival

The shrimp survival in the treatments exposed to single and combination of pH and TAN stressor is shown in Fig. 1. All the treatments showed significant differences (p ≤ 0.01) with Log-rank (Mantel-Cox) test and Log rank test for trend. At the end of the stress experiment, individual TAN and pH treatments recoded survival (%) as: TAN1 (95) > TAN3 (75) > TAN6 (70) > TAN9 (55) and pH8 (95) < pH6 (70) < pH10 (50). Among the combined treatments, survivals were:

Table 1
Results of Cox's proportional hazard factor EXP (B) for both individual (pH and TAN) and combination treatment (pH X TAN) using post stress survival as explanatory variable and the control being used as reference.

Treatment	B	SE	NH_3-N	NO_2-N	P(Sig.)	Exp(B)	95.0% CI for Exp(B)		Median Survival
			(mg/l)	(mg/l)			Lower	Upper	Days
Control	–	–	0.001	0.000	0		–	–	#
pH6*	1.947	1.08	0.001	0.000	0.071	7.0	0.844	58.2	#
pH8	0.002	1.414	0.001	0.000	0.999	1	0.063	16	#
PH10*	2.519	1.049	0.001	0.000	0.016	12.4	1.588	97	14
TAN1	0.025	1.414	0.020	0.001	0.986	1	0.064	16.4	#
TAN3	1.775	1.095	0.060	0.002	0.105	5.9	0.689	50.5	#
TAN6	2.008	1.08	0.120	0.003	0.063	7.5	0.897	61.9	#
TAN9*	2.584	1.054	0.180	0.004	0.014	13.3	1.678	104.6	#
pH6TAN1*	3.23	1.031	0.002	0.001	0.002	25.3	3.349	190.7	11
pH6TAN3*	2.713	1.041	0.007	0.022	0.009	15.1	1.96	116	13
pH6TAN6*	2.803	1.038	0.016	0.066	0.007	16.5	2.158	126.2	13
pH6TAN9*	3.449	1.03	0.017	0.360	0.001	31.5	4.18	237.1	10
pH8TAN1	0.017	1.025	0.069	0.231	0.99	1	0.064	16.3	#
pH8TAN3*	2.528	1.054	0.290	0.363	0.016	12.5	1.587	98.9	#
pH8TAN6*	2.648	1.049	0.360	0.418	0.012	14.1	1.808	110.3	14
pH8TAN9*	2.859	1.041	0.700	0.480	0.006	17.4	2.268	134.2	13
pH10TAN1*	2.906	1.038	0.880	0.219	0.005	18.3	2.391	139.8	13
pH10TAN3*	4.202	1.03	1.550	0.298	0.001	66.8	8.861	503.7	6
pH10TAN6*	8.996	1.433	2.570	0.400	0.001	8071.2	486.506	133902	1
pH10TAN9*	8.996	1.433	2.930	0.500	0.001	8071.2	486.506	133902	1

SE standard error of coefficient 'B', P value indicates statistical significance, Exp (B) exponential or antilog of coefficient 'B', 95% CI for Exp (B) the interval in which the true value of Exp (B) lies with 95% confidence. * indicates $P \leq 0.05$. # indicates undefined median survival i.e., 50% of shrimp survived in that treatment.

pH6TAN3 (40), pH6TAN6 (35) pH6TAN1 (20), pH6TAN9 (15), pH8TAN1 (95), pH8TAN3 (55), pH8TAN6 (45), pH8TAN9 (40), pH10TAN1 (35), pH10TAN3 (15). The combination treatments, pH10TAN6 and pH10TAN9 showed 100% mortality within 24 h from the start of the experiment.

Median survival reflects the time after which 50% of shrimp have survived after exposure to individual and combination of stressors (Table 1). Maximum median survival of 14 days was observed in pH10 and pH8TAN6 followed by 13 days in pH6TAN6, pH6TAN3, pH8TAN9 and pH10TAN1, 11 days in pH6TAN1, 10 days in pH6TAN9, 6 days in pH10TAN3 and one day in pH10TAN6 and pH10TAN9.

Cox's proportional hazard model is analogous to a multiple regression model, enabling the difference between survival times of particular treatments to be tested while allowing for other factors. In this model, the survival which is the dependent variable constitutes the 'hazard'. The hazard is the probability of dying or risk of dying i.e., shrimp survival up to a given point of time after stressor exposure. The probability of risk factor EXP (B) varied from 1 to 13 times in individual pH and TAN treatments. The highest risk factor was in TAN9 (13.25) followed by pH10 (12.41). Among the combined treatments, pH6, pH8 and pH10 with TAN showed 15 (pH6TAN3) to 31 times (pH6TAN9), 1 (pH8TAN1) to 17 times (pH8TAN9) and 18 (pH10TAN1) to 8071 (pH10TAN6 and pH10TAN9) times respectively, increased risk of dying compared to control.

3.2. Effect of pH and TAN stress on water parameters

Nitrite-N (mg/l) concentration increased proportionately with TAN concentration in all the individual TAN treatments (Table 1). There was no variation among the three pH individual treatments, and an increase was observed in all the combination treatments compared to control. Unionized ammonia was calculated based on the temperature, salinity and pH. In individual TAN treatments, the toxic form of unionized ammonia increased proportionately with the TAN levels. However, the values were significantly less ($P \leq 0.05$) in individual treatments compared to combined treatments (Table 1). Among the combination treatments, high concentration of unionized ammonia (mg/l) was registered in pH10TAN9 (2.931) and lowest in pH6TAN1 (0.002). In all the combined treatments it increased significantly ($P \leq 0.05$) with the increase in pH and TAN concentration.

3.3. Effect of pH and TAN stress on gills histopathology

The samples for histopathology included all treatments except pH10TAN6 and pH10TAN9 where 100% mortality was observed on day-1. The results revealed moderate to severe structural changes in all treatments, whereas no structural abnormality of gill was evident in the control (Fig. 2A). There were severe cellular architectural alterations in the gills of shrimp exposed to altered pH (either acidic or alkaline). These histological changes included hemocyte infiltration, edema, indicative of inflammation, lifting of lamellar epithelium, fused lamellae and disrupted pillar cells resulted in increased interlamellar space, hyperplasia, epithelial damage including sloughing and necrosis (Fig. 2B and C). Histology of the gills of shrimp exposed to the different levels of pH and TAN showed various degrees of lesions except in those at pH8. Hemocyte infiltration, edema, hyperplasia, fused lamellae and necrosis was observed in gills of shrimp exposed to TAN9 (Fig. 2D), and in other treatments change in gill structures indicated progressive destruction of epithelia. In pH6TAN1, pH6TAN9 and pH8TAN9 the lesions included thickening of the lamellar epithelium, lifting of lamellar epithelium, fusion of secondary lamellae, rupture of capillaries, cellular edema, cellular tumefaction and hyperplasia (Fig. 2E–G). Shrimp exposed to pH6TAN9 and pH10TAN3 exhibited enhanced gill damage, seen as the high degree of degeneration of the gill architecture that includes hemocytic infiltration, cellular tumefaction, sloughing of lamellar epithelium, desquamation of lamellae and necrosis, could partly be attributed to the higher incidence of mortality (Fig. 2H and I).

3.4. Effect of pH and TAN stress on immune variables

3.4.1. Effect of pH and TAN stress on THC

After 24 h of stress experiment, THC (x 10^5 cells/ml) significantly decreased in all the treatments compared to control (Fig. 3A). Among the combination treatments, the highest THC was noted in pH8TAN1 (147) on day-1 and the lowest count in pH6TAN9 (51.2) on day-7. By day-14, amongst the combination treatments, significantly less THC was observed in pH6TAN9 (53) in pH6 group and pH8TAN9 (61) in pH8 group and pH10TAN3 (61.5) in pH10 group. Between pH

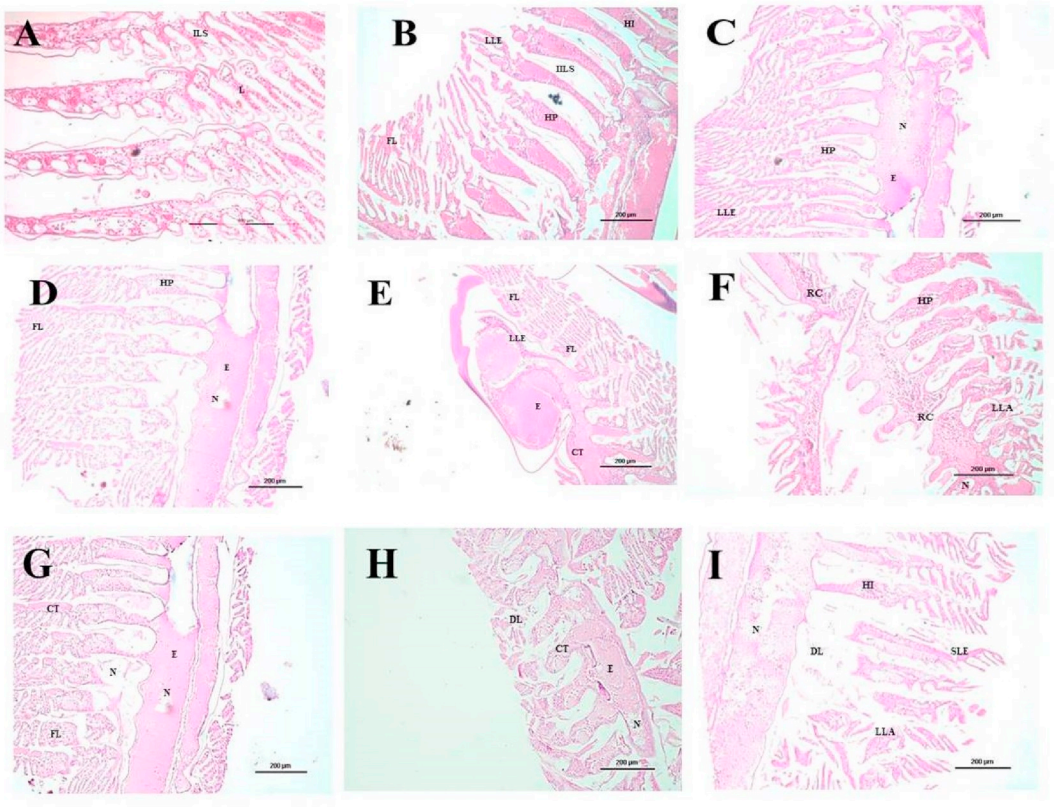

Fig. 2. Histopathology (H&E-stained sections 10X) of gills from *Penaeus vannamei* subjected to different stress treatments. (A) Gill from control (B) Gills exposed to pH6 (C) Gills exposed to pH10 (D) Gills exposed to TAN9 (E) Gills exposed to pH6TAN1 (F) Gills exposed to pH6TAN9 (G) Gills exposed to pH8TAN9 (H) Gills exposed to pH10TAN1 (I) Gills exposed to pH10TAN3 showing Fused lamellae (FL); Hemocytes (H); Hemocytic infiltration (HI); Hyperplasia (HP); Inter lamellar space (ILS); Increase Interlamellar space (IILS); Lamella (L); Lifting of the lamellar epithelium (LLE); Necrosis (N); Cellular tumefaction (CT); Edema (E); Loss of the lamellar architecture (LLA); sloughing of the Lamellar epithelium (SLE); Desquamation of Lamellae (DL); Rupture of capillaries (RC).

individual treatments, pH6 and pH10 treatments showed less hemocyte count which decreased with days of exposure compared to pH8 treatment. The THC was inversely proportional to the TAN concentration and exposure time in individual and combined TAN treatments.

3.4.2. Effect of pH and TAN stress on PO activity

Individual stressors (pH and TAN) effect on shrimp showed significantly less PO activity (U/ml) from day-1 to day-14 except in pH8 and TAN1. However, a similar trend was not discernible in the combination treatments (Fig. 3B). The highest PO (U/ml) activity was observed in pH6TAN1 (0.397) and lowest in pH10TAN3 (0.175). Among the pH6 combination treatments pH6TAN1, pH6TAN3 pH6TAN6 and pH6TAN9 registered PO activity of 0.3695, 0.3341, 0.2019 and 0.1747 on day-1 which decreased to 0.1349, 0.1568, 0.1417 and 0.1097 respectively by day-14. Treatment combinations of pH8 showed a significant decrease in PO from day-1 to day-14 of stress experiment except pH8TAN1.The lowest PO activity was seen in pH8TAN9 on day-1 (0.247) and 50% of its activity was reduced by day 14 (0.123). Among the combination treatments a significantly lower PO activity was recorded in pH10 compared to pH6 and pH8. The PO activity in pH10TAN1 and pH10TAN3 showed 0.148 and 0.181 on day-1 and reduced further with exposure time and recorded significantly less activity of 0.1096 and 0.0567 respectively on day-14. By the end of the stress experiment PO activity reduced by 80 and 90% in pH10TAN1 and pH10TAN3 respectively compared to control.

3.4.3. Effect of pH and TAN stress on SOD activity

The SOD (U/ml) decreased significantly ($P \leq 0.05$) in all the treatments except in pH8 and TAN1 compared to control (Fig. 3C). In individual treatments, enhanced activity was observed in pH8 compared to pH6 and pH10 irrespective of the days of exposure. Among individual TAN treatments, SOD decreased with increasing concentration as well as exposure time and significantly less value was recorded at the end. However, in case of combination treatments, a similar trend was not noticed and the SOD activity decreased from day-1 to day-7 which got restored from day-7 to day-14. Among the treatments of pH6 and its combination, highest SOD activity was recorded in pH6 (0.155) and lowest in pH6TAN9 (0.018). In pH8 and its combinations, the SOD activity of shrimp decreased significantly on day-7 and the lowest value observed in pH8TAN9 (0.014) on day-7 which was restored to 0.0894 on day-14. Among the pH10 treatment combinations, significantly high activity was observed in pH10TAN1 (0.098) and low in pH10TAN3 (0.008) on 14th day. In general, it was observed that the THC, PO and SOD activity followed more or less a similar trend and decreased with the increase in TAN levels. Less immune activity was seen in combined treatments compared to individual treatments.

3.5. 5 Effect of pH and TAN stress on susceptibility to WSSV

Survival data was recorded from the post stress WSSV challenge experiment (Fig. 4). All the treatments of pH and TAN significantly differed in mortality ($P \leq 0.01$) compared to control with log rank (Mantel-Cox) test and log rank test for trend. Among individual treatments, mortality of the shrimp started on 2nd day after challenge and 100% mortality was seen on 6th day at pH10, 7th day at pH6, and 9th day at pH8 and control. All the shrimp died in TAN9 by 6th day, TAN3 and TAN6 by 7th day and TAN1 and control by 9th day. All the pH6 combinations irrespective of TAN concentration exhibited mortality by day-1 post challenge and all shrimp died by 4th day. In pH8 combination, mortality started on the 4th day of post challenge whereas at

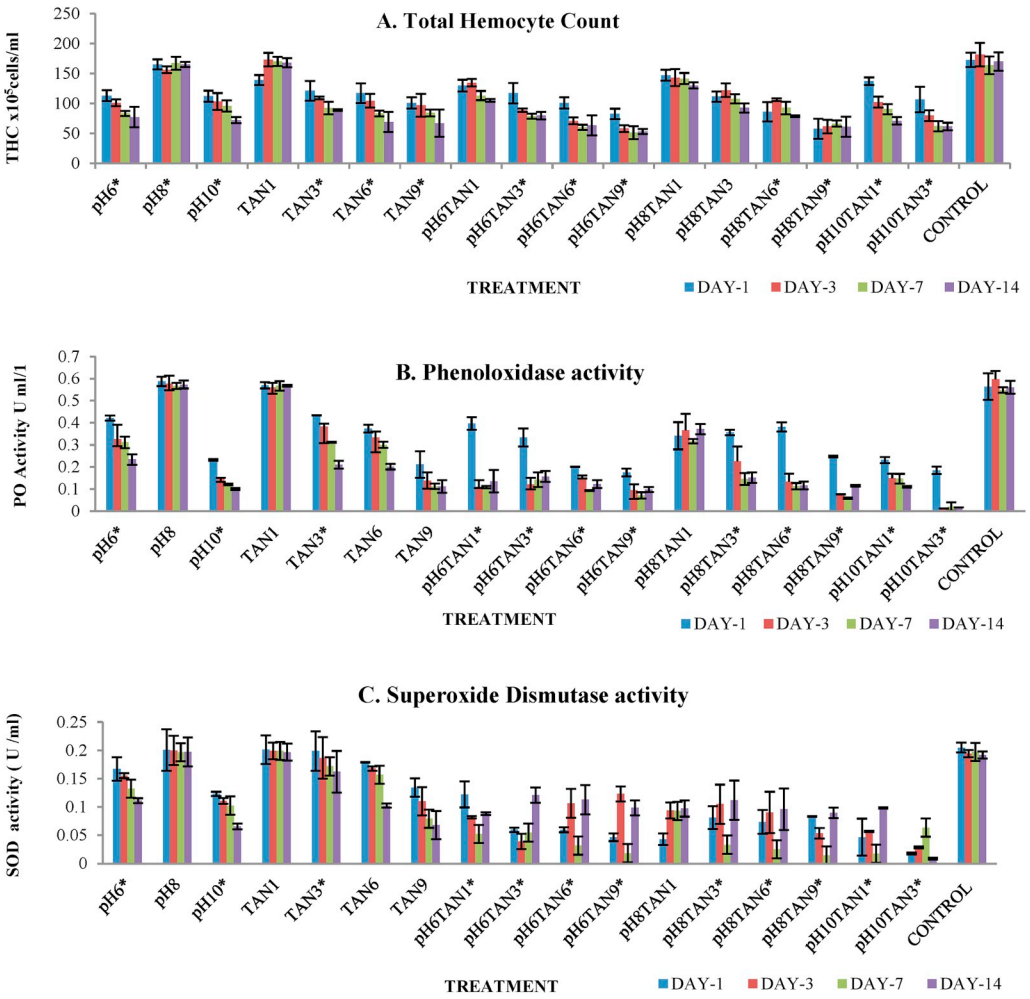

Fig. 3. Effect of pH and TAN stress in *P.vannamei* on (A) Total hemocyte count [x 10^5 cells/ml] (B) Phenoloxidase activity (U/ml) (C) Superoxide Dismutase activity (U/ml). Bars represent means ± SD. * indicates significance at P ≤ 0.05.

pH6 and pH10 the shrimp died from 2nd day onwards in combined treatments. 100% mortality was observed on 9th and 7th day in pH8TAN1 and pH8TAN3 respectively whereas, pH8TAN6 and pH8TAN9 showed 100% mortality by 5th day and 4th day respectively. Among the pH10 combination treatments, pH10TAN3 showed 100% mortality by 2nd day whereas all shrimps died on 5th day in pH10TAN1.

The observed clinical signs of WSSV infection included reduced feed consumption, reddish discoloration of the body that eventually led to mortalities in all treatments. WSSV infection was confirmed by PCR and all the tissue samples from treatment groups were found first step positive for WSSV (Fig. 5).

Cox proportional hazard Exp (B) value, the risk of dying and median survival after post WSSV infection is depicted in Table 2. Among the individual treatments of pH and TAN, the possibility of dying was 0.9–5.3 times more compared to control. Among the individual treatments, highest median survival of 6 and 5.5 was recorded in pH8 and TAN1, respectively whereas pH10 and TAN9 recorded lowest median survival of 3.5 and 4 days. Among the combination treatments of pH8, the risk of dying was about 1.4–9.9 times more compared to control and recorded median survival of 3–5.5 days. The pH6TAN1, pH6TAN6, pH6TAN9 and pH10TAN3 treatments showed a median survival of 2 days, and the probability of dying varied from 14.9 to 35.6 times. Higher threat was recorded in pH10TAN3 and pH6TAN9 with 36 and 28 times more risk of dying (P ≤ 0.05) compared to control.

4. Discussion

4.1. Effect of pH and TAN stress on survival

TAN levels above of 1 mg/l can interrupt the body metabolism, negatively affect the performance and cause death in shrimp (Magallón Barajas et al., 2006). Individual TAN treatments (1, 3, 6 and 9 mg/l) exhibited survival of 95, 75, 70 and 55% respectively after 14 days whereas the combination treatments, pH8TAN1 and pH8TAN9 showed 95 and 40% survival indicating that TAN has indeed a prominent role in buildup of toxicity thereby causing mortality. These results corroborate the findings of Liu and Chen (2004) who reported cumulative mortality of 63.3 and 66.7% in *P. vannamei* exposed to 11.1 and 21.6 mg/l of TAN in 7 days.

It has been studied that low or high water pH can impair survival, ion-transport, enzyme activities, reduce immunity, weaken antioxidant ability and cause DNA damage in *P. vannamei* (Li and Chen, 2008, Magallón Barajas et al., 2006; Pan et al., 2007; Wang et al., 2002; Zhou et al., 2009). However decapod crustaceans have the ability to tolerate basic and slightly acidic conditions due to the exchange of Na^+/H^+ and Cl^-/HCO_3^- across their gills (Henry et al., 1981). Wang et al. (2002) observed that *P. chinensis* reared in pH 6.0 and pH 8.5 environments experienced significantly higher mortality compared to those at pH 7.6 after 14 days. In the present study, the mortality in individual pH10 treatment started on 9th day after commencement of experiment and showed 50% mortality by 14th day. The results are in agreement with

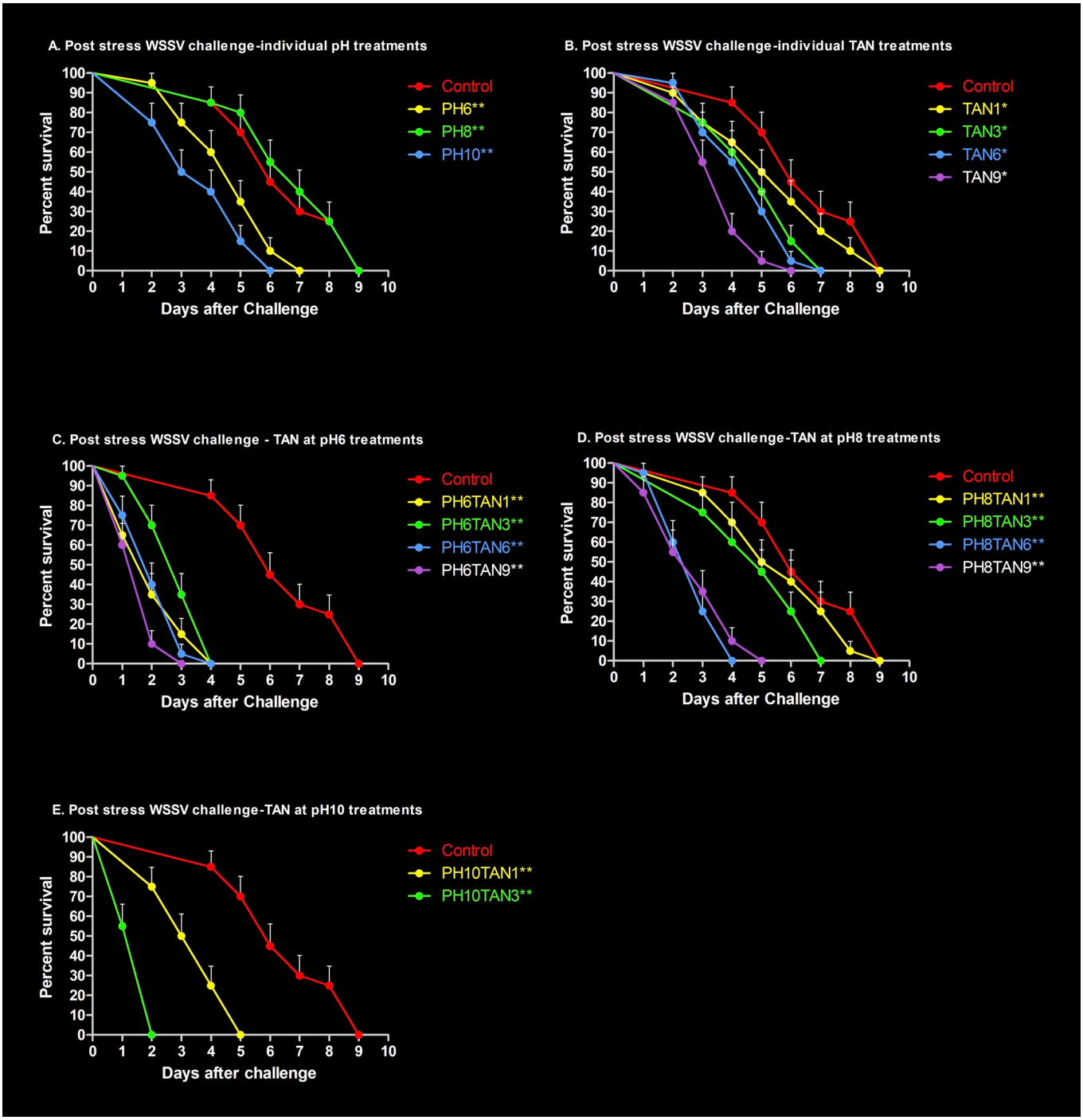

Fig. 4. Shrimp survival % (Mean ± SE) of the pH and TAN individual and combined stressor treatments post WSSV challenge. (A) Post stress WSSV challenge-individual pH (b) Post stress WSSV challenge-individual TAN (C) Post stress WSSV challenge-TAN at pH6 (D) Post stress WSSV challenge - TAN at pH8 (E) Post stress WSSV challenge-TAN at pH10. ** indicates $P \leq 0.01$.

those reported by Li and Chen (2008), where shrimp exposed to high pH (10.1) showed no mortality up to 7 days. The shrimp reared in pH6 started dying on 6th day after stressors exposure and showed better survival (70%) compared to pH10 (50%) with the risk hazard of 7.14 and 13.8 times in pH6 and pH10 respectively. Zhou et al. (2009) also found a higher mortality in juvenile *P. vannamei*, when exposed to pH 9.3 for 12 h than in those exposed to pH 5.6, where hemolymph pH increased significantly after exposure to pH 9.3, indicating that this species has lower tolerance to basic pH (10.0) than acidic pH (4.5) (Wang et al., 2002; Zhou et al., 2009).

Stress factors that are present in combinations pose a far greater threat than individually (Plumb, 1991). Increase in pH levels favor the formation of the more toxic un-ionized form of ammonia and enhance the toxic effects (Colt and Armstrong, 1981). A similar pattern was observed in our study with increased levels of toxic forms of unionized ammonia in pH10 treatment. Among the treatment combinations of pH10, the TAN toxicity increased 448 times more from TAN1 to TAN9 and resulted in higher risk of dying (18–8071 times). In a similar study by Magallón Barajas et al. (2006) in *P. vannamei* post larvae after 4 h exposure to pH and ammonia levels, survival reduced significantly in the combined treatment of ammonia-N (7 mg/l) at pH 9. Increase in pH levels increases NH_3-N concentration, which leads to the diffusion and

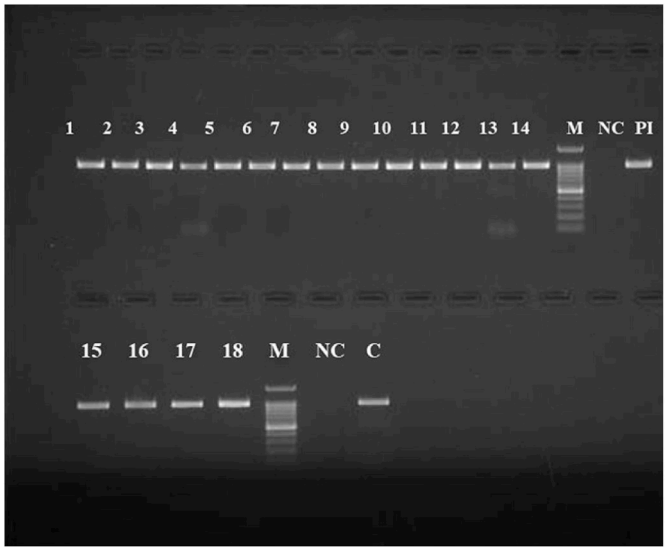

Fig. 5. Detection of WSSV in the gill and pleopods tissue of stressed *P. vannamei* shrimp by PCR. Lane M: 100bp Marker Lane 16&22: NC: Negative control; PI: WSSV Positive Inoculum**; C**-Control; Lane 1–18 samples found first step positive (Lane 1–3: pH6, pH8, pH10; Lane 4–7: TAN1, TAN3, TAN6, TAN9; Lane 8–12: pH6TAN1, pH6TAN3, pH6TAN6, pH6TAN9; Lane 13–16: pH8TAN1, pH8TAN3, pH8TAN6, pH8TAN9; Lane 17–18: pH10TAN1, pH10TAN3).

accumulation of ammonia in hemolymph, which even at lower concentrations can be toxic for short-term exposure (Chen and Sheu, 1989). Similar works have been reported on the short-term effects of the combination of stressors on shrimp survival (Allan et al., 1990; Chen and Chen., 1996; Chen and Lin, 1992; Kir et al., 2004; Lin and Chen, 2001; Martînez et al., 1998; Parado-Estepa, 1998; Wajsbrot et al., 1990; Zhang et al., 2006). The present results do not throw light on the mechanisms of toxicity. In pH6TAN1 the mortality of shrimps, might partly be attributed to the increased loss of sodium ions through the gills at low pH levels to regulate internal acid-base status (Duarte et al., 2013) where Na^+ can exchange with NH_4^+/H^+ ions. Low pH causes damage to the gill tissues of aquatic animals because crustaceans regulate internal pH to some extent through Cl^-/HCO_3^- and Na^+/H^+ exchanges via the gills (Henry et al., 1981; Wickins, 1984) and this caused impaired ionic regulation. Young-Lai et al. (1991) observed that lower hemolymph sodium concentrations and ammonia in the external medium could affect the Na^+/NH_4^+ transport mechanism by impairing the transport sites for sodium. At high toxic ammonia concentrations, NH_3 infuses cell membranes quite easily, affecting intracellular pH and transamination reactions (Colt and Armstrong, 1981; Chen and Lei, 1990; Hargreaves and Tucker, 2004). Based on the analysis of Wright and Wood (1985), acid exposure should raise the diffusional gradients for both NH_3 and NH_4^+, thereby increasing ammonia excretion. Hence in the present study, the lower survival of 15% in pH6TAN9 is probably the outcome of more diffusional gradients that might affect the physiology of the shrimp. The combination of TAN9 with pH 6, 8 and 10 showed 15, 40 and 0% survival which may be due to the enhanced toxicity of ammonia from pH6 to pH10. The combination of these pH levels at TAN3 i.e., pH6TAN3 and pH10TAN3 showed 15 and 66 times more risk hazard while individual TAN3 showed 6 times more risk of dying compared to control. This could be related to the findings that toxicity of ammonia at any particular concentration increases with increasing pH level (Noor-Hamid et al., 1994). According to Chen and Lin (1992) the maximum acceptable concentration of toxic ammonia (NH_3) for penaeid shrimp is 2 mg/l and in our study, NH_3 was more than 2 mg/l which might have caused acute mortality in the combination treatments pH10TAN6 and pH10TAN9.

4.2. Effect of pH and TAN stress on water quality parameters

In our study, the correlation coefficients were 0.42 between TAN and NH_3 and 0.86 between pH and unionized ammonia which implies a strong positive correlation between pH and NH_3 than TAN and NH_3 indicating that the ammonia-N has a more toxic form (un-ionized ammonia) at high pH and a less toxic form (ionized ammonia, NH_4^+) at low pH. This study supported the contention that $NH4^+$:NH_3 ratio is strongly pH-dependent and ammonia toxicity increases at a higher pH since NH_3 prevails over NH_4^+ (Armstrong et al., 1978), whereas salinity and temperature exert a lesser effect (Lin and Chen, 2001; Romano and Zeng, 2010). The ratio of unionized ammonia to ammonium ion increases 10-fold for each unit increase in pH (Boyd, 1982; Erickson, 1985). Among the water parameters, the correlation between nitrite-N and TAN (0.62), and pH (0.65) clearly signifies the linear and positive relation between these variables and nitrite–N increased from pH6 to pH8 and also with increase in TAN concentration in all the treatments.

Table 2
Results of Cox's regression for varying individual (pH and TAN) and combination treatments (pH X TAN) using survival post WSSV challenge as explanatory variable and control as reference. Median survival time is denoted in days.

Treatment	Coefficient	SE	P value	Exp(B)	95.0% CI for Exp(B)		Median Survival
	B				Lower	Upper	Days
Control	–	–	0.000	–	–	–	7
pH6*	0.962	0.331	0.004	2.6	1.369	5.002	5
pH8	−0.093	0.316	0.769	0.91	0.49	1.694	6
PH10*	1.46	0.335	0.000	4.3	2.234	8.307	3.5
TAN1	0.388	0.318	0.222	1.4	0.791	2.746	5.5
TAN3*	0.88	0.33	0.008	2.4	1.263	4.598	5
TAN6*	1.078	0.332	0.001	2.9	1.533	5.631	5
TAN9*	1.669	0.339	0.000	5.3	2.732	10.313	4
pH6TAN1*	2.703	0.348	0.000	14.9	7.542	29.516	2
pH6TAN3*	2.147	0.347	0.000	8.6	4.342	16.889	3
pH6TAN6*	2.751	0.351	0.000	15.6	7.868	31.135	2
pH6TAN9*	3.329	0.358	0.000	27.9	13.821	56.315	2
pH8TAN1	0.369	0.32	0.249	1.4	0.772	2.711	5.5
pH8TAN3*	0.773	0.329	0.019	2.1	1.137	4.123	5
pH8TAN6*	2.291	0.347	0.000	9.8	5.004	19.519	3
pH8TAN9*	2.128	0.343	0.000	8.4	4.292	16.441	3
pH10TAN1*	1.759	0.341	0.000	5.8	2.978	11.319	3.5
pH10TAN3*	3.574	0.365	0.000	35.6	17.431	72.916	2

SE standard error of coefficient 'B', P value indicates statistical significance, Exp (B) exponential or antilog of coefficient 'B', 95% CI for Exp (B) the interval in which the true value of Exp (B) lies with 95% confidence. * indicates ($P \leq 0.05$).

The action of nitrite on the respiratory pigments and their ability to capture and transport oxygen in the hemolymph could cause a decrease in the aerobic metabolism and consequently a decrease in the food consumption rates (De Campos et al., 2014). High levels of nitrite-N causes stress in animals and in our study, high levels of nitrite-N observed in pH10 combination treatments might have increased the stress. Such a view was proposed by Jensen (1996), wherein he reported that the prime toxic actions of nitrite are possibly due to oxidation of heamocyanin to metaheamocyanin. Under high nitrite environment, nitrite competes with the oxygen active site of copper and converts heamocyanin into metaheamocyanin which is nonfunctional, and ineffective to transfer the amount of O_2 that is available for metabolism. The current study clearly signifies that high levels of nitrite-N and toxic form of ammonia (NH_3) in water are the plausible causes for acute mortality of shrimp in pH10TAN6 & pH10TAN9.

4.3. Effect of pH and TAN stress on gills histopathology

Gill pathology studies suggest that varying concentrations of TAN and pH, as well as their combinations, trigger alterations in gill structure. Therefore, the consequent moderate to severe tissue damage could have induced high mortality (85%) in shrimps exposed to pH6TAN9 and pH10TAN3 combinations. The major part of ammonia is excreted mainly through the gill epithelium (Kinne, 1976; Regnault, 1987) through diffusion of NH_3 and NH_4^+ and Na/NH_4^+ exchanges (Péqueux and Gilles, 1981; Pressley et al., 1981). The NH_3 molecule is highly toxic due to lipophilic nature and lack of charge, crosses cell membranes of gills more rapidly (Campbell and Jones, 1990; Chen and Kou, 1993; Randall and Wright, 1987) than the charged and hydrated NH_4^+ ions (Downing and Merkens, 1955; Tomasso, 1994; Wuhrmann and Woker, 1949). The findings in our study are in agreement with those reported by Weihrauch et al. (2009) wherein they observed that an increased amount of ammonia accumulation ultimately leads to stress and acute mortalities of shrimp as seen in the combinations pH10TAN6 and pH10TAN9. Slightly acidic (6.0) or basic (8.5) water pH resulted in metabolic distress, inhibiting the activity of Na^+–K^+ ATPase in *F. chinensis* (Wang et al., 2002). Thus impairment of the active transport mechanism for sodium ions through the gill epithelium is the primary cause of the death of shrimp in acid and alkaline water (Wang et al., 2002). Nitrite and ammonia are considered to be taken up directly by the animals and accumulated in the hemolymph through a mechanism associated with the chloride cells of the gills (Laurent and Dunel, 1980). Nitrogenous compounds can injure the gill tissue, affecting the oxygen transport and cause mortality of the rearing organisms (Barbieri et al., 2016; De Campos et al., 2014; Lin and Chen, 2001, 2003). From the above studies, it is evident that acidic and basic pH coupled with high TAN levels are known to cause cellular destruction of gills. Our results evidenced the epithelial damage of gills that might impair the osmotic regulation and possibly resulted in low survival in individual stressor treatments. Also the effect might be additive and caused severe mortality in combination treatments pH6TAN9 and pH10TAN3 that was evident from the extensive cellular pathology study of gills. .

4.4. Effect of pH and TAN stress on immune variables

Extrinsic factors like temperature, salinity, pH and ammonia affect circulating hemocytes and immune variables in several species of decapod crustaceans (Cheng et al., 2003; Jiang et al., 2004; Wang and Chen, 2005). Chen et al. (2015) stated that wide variations in environmental factors might induce changes in the immune system in *P.vannamei* that are often immunosuppressive, by diminishing the total hemocyte number and decreased PO and SOD activity, thereby increasing the risk of susceptibility to infectious diseases. The results in the present investigation have shown that all the three immune variables studied, viz., THC, PO and SOD activity were significantly affected after exposure of the shrimp in the individual as well as the combination treatments of pH and TAN. Similarly a significant reduction in the immunological activity of *P.vannamei* (Li and Chen, 2008) when exposed to low (6.5) and high pH (10.1) and considerably lower PO and THC at pH 4·6-5·0 and 9·0–9·5 were reported in *M. rosenbergii* (Cheng and Chen, 2002). Environmental distresses due to ammonia (Pan et al., 2003), nitrite-N (Xian et al., 2011), acute changes in acidic pH (Wang et al., 2009) and chronic high pH (Han et al., 2018) can elicit an increase in the production of reactive oxygen species that induce oxidative stress and reduce super oxide dismutase activity (Di Mascio et al., 1991). Our results indicating significantly lower SOD activity in both individual and combined stressor treatments are in agreement with the findings reported above. Le Moullac and Haffner (2000) reported 30% decrease of THC following exposure to ammonia at 3 mg/l in *P. stylirostris*. The PO activity of *M. rosenbergii* was significantly lower after seven days of exposure to ammonia-N at 0.55, 1.68 and 3.18 mg/l (Cheng and Chen, 2002). The present findings are also similar to the outcome of the above studies as immunological activity was significantly low in pH6 and pH10 compared to pH8 treatment and THC, PO and SOD activity decreased with increase in TAN in individual and combined treatments especially at high pH and TAN levels, reducing the immune competence leading to low survival and consequent mortality.

4.5. Effect of pH and TAN stress on susceptibility of shrimp to WSSV

Changes in water physico-chemical parameters are considered as WSSV risk factors because changes in pond water quality increase the stress and susceptibility to the disease in *P. vannamei* (Guan et al., 2003; Peinado-Guevara and López-Meyer, 2006; Rahman et al., 2006). Under stressful conditions, WSSV can multiply rapidly and cause mortality (Doan et al., 2009; Lo and Kou, 1998). Also stress due to elevated TAN and pH caused reduction of THC, PO and SOD in *Penaeus japonicus* (Chen and Kou, 1991) and *Penaeus chinensis* (Sun and Ding, 1999). Acute mortalities in the present study were linked to exposure of animals to the combined stressor (pH and TAN) that enhanced risk of dying in shrimp challenged with WSSV. This is because of exposure of shrimp to stressor increased the risk of WSSV, since stressors compromise the shrimp immune system (Chen and He, 2019). Chen et al. (2010) and Zhang et al. (2016) studies on *P.vannamei* also confirm that the changes in ammonia-N levels and pH are likely to trigger the disease outbreak. Post stress WSSV challenge trials in our study also confirmed the enhanced susceptibility to WSSV due to increased viral proliferation under stress conditions which was proven by the positive first step PCR. Among the molecular diagnostic methods used for aquatic animal disease diagnosis, PCR is the gold standard and nested PCR is used to screen brood stock and post larvae before stocking the ponds (Hsu et al., 1999). It is generally accepted that nested PCR amplification methods are more sensitive for the detection of WSSV from infected shrimp tissues (Lo et al., 1996a, 1996b). The nested PCR allows the gradation of viral infection (Ayub et al., 2008; Lo et al., 1996b) and the samples positive by first step are highly infected by WSSV and those positive only by nested step indicated a lower level of infection. Though the nested PCR is not quantitative, it is reported that first step of the PCR assay roughly corresponds to a detection range of 10,000 or more virions/g of tissue and the second step PCR is known to detect the range of 500–2500 virions/g of tissue (Sritunyalucksana et al., 2006).

In our study, nested PCR revealed a heavy viral load in tissues of experimentally infected shrimp. Increased viral replication is directly linked to the presence of a combination of stressors (Jeswin et al., 2015). Though the viral load is a critical parameter, other factors also influence the spread of the WSSV and subsequent mortalities. The outcome of our study suggests that the combination of viral load and the stressor combinations determine the acute shrimp mortalities which are expressed in terms of risk of dying Exp (B) factor.

Cox proportional hazard model revealed that shrimps exposed to individual pH10 and TAN3 showed 5.81 and 2.41 times risk of dying,

whereas pH10TAN3 showed 35.64 times risk of dying compared to control. It is clear that the combined effect of pH10TAN3 is about 6 times more risk than pH10 and 15 times more than TAN3. The shrimps exposed to pH6TAN9 had higher risk of dying (27.9) than pH8TAN9 (8.4). The toxicity of ammonia in pH6TAN9 might not show the same interaction effect in pH8TAN combination treatments. A reduction in pH from 8.2 to 6.5 or an increase to 10.1 made the shrimp more susceptible to *Vibrio alginolyticus* with lower immunological activities compared to those raised at pH 8.2 (Li and Chen, 2008). A similar phenomenon was observed in our study where the risk factor of 2.6 and 4.3 times more in pH6 and pH10 respectively compared to control leads to more susceptibility to WSSV compared to pH8 (0.9 times). Variations of pH (Corsin et al., 2001) and ammonia-N (Xue et al., 2017) reduced immuno-competence to WSSV and triggered the disease outbreak. This aspect is in line with our observations which revealed early mortality of shrimp with WSSV infection in treatments compared to control. Fouzi et al. (2012) also showed that exposure of *P.monodon* to higher ammonia (1.1, 3.8 and 8.1 mg/l) caused stress and subsequent infection with WSSV led to early mortality. High pH (8.8–9.7) and low temperatures influenced WSSV infection in cultured shrimp *P. monodon* (Gunalan et al., 2010). Jiang et al. (2004) reported severe immunological compromise for the post ammonia stressed shrimp challenged with WSSV. To the best of our knowledge, this is the first report from India on the effect of ammonia stress on *P.vannamei* under varying levels of pH and post stress challenge studies with White Spot Syndrome Virus.

5. Conclusions

Our study demonstrated more tangible effects of TAN coupled with pH compared to the impact of TAN or pH alone. The combination of stressors resulted in the loss of self-adaptive ability after two-week exposure compared to individual treatments, the core cause for susceptibility to WSSV and subsequent mortality of shrimp. As there is no treatment for WSD, disease prevention by minimizing the stress factors emphasizes the need for maintenance of optimal environmental conditions. In view of the interaction effect of TAN and pH on survival, immune variables, histological changes in gills and susceptibility to WSSV in the present study, combined environmental factors rather than individual are vital for better management of the pond ecosystem.

Declarations of interest

None.

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