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Stress Quantification in *Penaeus vannamei* Exposed to Varying Levels of Turbidity

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ABSTRACT

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Increased water turbidity in the intensive farming of *Penaeus vannamei* is one of the environmental stressors. Investigations were carried out on the effect of inorganic clay particle induced turbidity (NTU) levels (T1-30, T2-60 and T3-120) on survival, immunological response: total hemocyte count (THC), activity of phenoloxidase (PO) and superoxide dismutase (SOD), and stress metabolites: Osmolality (O), glucose (G) and lactate (L) in *P. vannamei* exposed to 21 days. T3 and T2 registered 20 and 10% mortality respectively compared to 5% in T1 and control. Shrimp in T1, T2, and T3 showed 1.1, 1.9 and 4 times more risk of dying compared to control. THC, PO, and SOD decreased significantly ($p \leq 0.05$) from day-1 to 21 in all the treatments compared to control. T3 showed significantly low immunological activity (THC: 119±2.8 x 10^5 cells ml^{-1}; PO: 0.369±0.001 U ml^{-1}; SOD: 0.230±0.001 U ml^{-1}) compared to control (THC: 167±13.4 x 10^5 cells ml^{-1}; PO: 0.546±0.002 U ml^{-1}; SOD: 0.284±0.001 U ml^{-1}). Physiological stress metabolites increased significantly with exposure time in T3 (G: 85.2±0.071 mg ml^{-1}; L: 8.2±0.007 mg dl^{-1}) compared to control (G: 77.2.2±0.163 mg ml^{-1}; L: 7.1±0.16 mg dl^{-1}). Osmolality was high in T3 (961.7±148.6 mOsm kg^{-1}) compared to control (858.3±3.4 mOsm kg^{-1}). High turbidity of >60 NTU in the rearing medium caused the gills blockage apparently as a compensating reaction to the disruption of osmotic and ionic balance. Shrimps exposed to T3 treatment showed intracellular deposits of the clay particles within the filament and lamellar epithelium of gills in Scanning Electron Microscopy (SEM) images. The present study concluded that <30 NTU water turbidity as safe level for *P. vannamei*

ADDITIONAL INDEX WORDS: *Turbidity stress, glucose, lactate, osmolality, gills,* Penaeus vannamei

INTRODUCTION

Turbidity is an important indicator of the amount of suspended sediment in the water, which ensures many adverse effects on aquatic animals. Phytoplankton is a desirable form of turbidity in sufficient amounts because it provides food for microscopic animals mainly zooplankton and improves water quality by producing dissolved oxygen and removing potentially toxic compounds such as ammonia. On the other hand, turbidity caused by clay particles is generally undesirable because turbid water does not allow enough sunlight to penetrate into the deeper layers, which means a decrease in plankton survival and decreased dissolved oxygen output. Additionally, oxygen is constantly consumed by the micro-biota in the mud, results in the decrease of oxygen concentration (Oberle *et al*., 2019). The higher the turbidity levels, the less light that can reach the bottom waters of the pond. These low dissolved oxygen levels result in decomposition and phytoplankton die off in the lower layers, initiating hypoxic conditions. This can cause declined survival of shrimps in the pond (Boyd, Torrans, and Tucker, 2018).

Digging and pushing, the common behavior of most penaeid species also significantly increases turbidity at the pond bottom (Lin *et al.,* 1992). However, intensification of production resulting in an increase in suspended solids concentration will also lead to an increase in dissolved wastes, such as unionized ammonia (Becke *et al.,* 2019). Water turbidity affects water temperature, as suspended particles in pond water absorb and scatter sunlight; results in temperature shoot up of pond water (Marcus *et al*., 1990). Warmer water cannot hold much dissolved oxygen and tends to have a lower concentration of dissolved oxygen than water with a green phytoplankton bloom. The current problem of turbidity is arising from the feed. Being cost effective and increased demand (Naylor *et al.,* 2009), fish meal and fish oil are substituted by plant alternatives in feeds (Glencross, Booth, and Allan, 2007; Ytrestoyl, Aas, and Asgard 2015). This replacement coincidently causes a less dense and more fragile composition of fish feces (Schumann, Brinker, and Friedrich, 2018; Unger and Brinker, 2013), considerably increasing fine suspended solids in aquaculture waters (Brinker and Friedrich, 2012). These less dense fine particles are considered harmful to fish health and performance (Bilotta and Brazier, 2008). In addition, with the trend toward intensification, aerators have become more widely operated, about 3.7 times greater than normal ponds (Engle *et al.,* 2017). Especially in

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earthen ponds, aeration can stir up bottom sediments and results in an increase of turbidity. Cultured Penaeid shrimps at juvenile and adult stages, which are benthic dwellers spend long periods in sediments, are more frequently exposed to high turbidity. In shrimps, gills are the principal site of respiration and play an essential role in the control of ion and water balance. The gills always links with rearing waters, offer the pathogen and toxins contact with the gill thus contributing to the maintenance of the organisms' physiological condition. Hence, gill is considered as one of the most important and vulnerable organs in diagnosing the health of a shrimp (Khoa, Hatai, and Aoki, 2004). Fine particles easily block the gill lamella, resulting in a shortage of dissolved oxygen, leading to death of fish (Viadero and Noblet, 2002). High concentrations of fine turbid clay particles can also clog gills causing stress in shrimps. Haemolymph metabolic components like glucose, lactate, osmolality, total hemocyte count (THC), phenoloxidase (PO) and superoxide dismutase (SOD) act as indicators of the physiological status of shrimps (Mercier *et al.,* 2006; Rosas *et al.,* 2004; Sanchez *et al.,* 2001).

Against this background, there is little information available on the physiological effects of turbidity stress in *P. vannamei*. Previous studies have demonstrated that high turbidity may impair reproduction and osmoregulation, reduce the exchange rate of O_2 and alter respiration in molluscs, fish and shrimp (Charmantier and Soyez, 1994; Daou and Goulletquer, 1988; Servizi, Gordon, and Martens, 1987). Servizi and Martens (1991) suggested that mortality in turbidity exposed fish is due to the reduced uptake or exchange of oxygen. To our knowledge, no study concerning the physiological effect of turbidity has been reported for crustaceans. In the present study, we aimed to investigate the direct impact of these accumulating turbid particles on stress and immunological performance on *P. vannamei*. We here report specifically different levels of turbidity and their impact on survival, immunological and metabolic variables of hemolymph and gills of *P. vannamei*.

METHODS

The methodology includes the detailed account of the procedure that was followed in completing the *P. vannamei* turbidity stress experiment with execution of acclimatization, experiment design followed by sampling and analysis of water and hemolymph.

Animal Collection and Maintenance

Healthy, Pacific white shrimp, weighing (8-10 g) each, were collected from a shrimp farm at Padappai, Tamil Nadu, India and acclimatized in the laboratory of CIBA HQ, Chennai for two weeks before conducting an experiment. Shrimp were kept in 1000L cement tanks with 700 L of filtered seawater (salinity: 20±2 ppt; temperature: 28.1±0.4°C; pH: 7.6±0.6). Shrimp were fed with standard feed pellets, equivalent to 3% of their body weight, twice a day.

Experiment Design

Shrimps weighed about 8.7±1.5g were exposed to different levels of turbidity (T30-30 NTU, T60-60NTU & T120-120 NTU) for three weeks in triplicate of 500 L containers with 30 shrimp in each treatment tank and control (turbidity-1±0.3 NTU). Water temperature during the experiment was maintained at 28.3±0.5°C, salinity-20 ppt and DO: 7.7 ±0.3 mg l^{-1}. All the treatment tanks were monitored on a daily basis for water turbidity, temperature, salinity, pH, total ammonia nitrogen (TAN) and nitrite-N using standard procedures (Clesceri *et al*., 1998). Water containing different levels of turbidity was made by first mixing a known amount of bentonite clay with water and aerating efficiently to keep the clay colloids in suspension (Ardjosoediro and Ramnarine, 2002). Turbidity was measured by Nephlometer (Elico CL-52D) twice daily and adjusted all the 3 turbidity levels accordingly by adding clay to increase the turbidity level or by adding water to decrease the turbidity level (T30- 30±3.7 NTU; T60- 60±4.1 NTU and T120- 120±5.0 NTU).

Sample Collection

Hemolymph samples collected from each experimental tank and control group (three random shrimps per treatment tank) on 1st, 7th, 14th and 21st day of experiment were separated into aliquots and processed for assessment of selected metabolic variables.

Immunological and Stress Metabolites Analysis

Total Hemocyte Count was evaluated by the method of Perazzolo *et al.* (2002). After thorough mixing of 20µL of anticoagulant-hemolymph, suspension was placed in a Neubauer slide (hemocytometer) and cells were counted using a phase contrast microscope to determine THC (cells/ ml hemolymph). Phenoloxidase activity was measured as for the methodology (Le Bris *et al*., 2015) by measuring L-dihydroxyphenylalanine (L-DOPA) absorbance due to the formation of dopachrome immediately read at 492 nm using a UV Spectrophotometer (Shimadzu UV-1700). The hemolymph SOD activity was determined following the method of Le Bris *et al*. (2015). The SOD activity was measured by reduction of water-soluble formazan dye, and SOD activity was assayed using the SOD assay kit (Sigma, 19160) following the manufacturer's instructions by recording the absorbance at 450 nm with UV-Spectrophotometer (Shimadzu UV-1700). Glucose was determined by the glucose oxidase method using a Sigma Aldrich kit (GAGO-20). Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Peroxidase acts as a catalyst, H_2O_2 reacts with o-dianisidine and H_2SO_4 results in the formation of a stable colored product. The intensity of the stable pink color was measured at 540 nm using UV Visible spectrophotometer (Shimadzu UV-1700). The glucose concentration was calculated from a standard curve of known glucose concentrations with a correlation coefficient value 0.9986. Lactate concentration was determined by an enzymatic colorimetric method using a lactate test Sigma Aldrich kit (MAK064). Lactate was first oxidized into pyruvate and then further peroxidized to form purple peroxidase products. All the procedures were protected from light. Each sample (2 µl) was mixed with 200 µl of lactate buffer and followed by 50 µl of master reagent mix per sample. After incubation at 25°C for 10 min, the absorbance of the sample was measured at 550 nm with a UV Visible spectrophotometer (Shimadzu UV-1700). A standard curve was generated from a set of lactate standards ranging from 0.0 to 0.2 mg/ml. Hemolymph osmolality (Lin *et al.,* 1992) was determined immediately after collection of

hemolymph sample with osmometer using Osmomat 3000, Gonotec, Germany.

Scanning Electron Microscope (SEM)

After three weeks of turbidity exposure, the whole shrimp were fixed in rapid fixative Davidson's Solution (Dewangan *et al*., 2015). After separation of gills, wet mount slides were prepared separately for all the treatments and control. These slides were then screened under Scanning Electron Microscope (JEOL JSM-IT300).

Statistical Analysis

Cox regression analysis was done and expressed as EXP (B) using SPSS version 21 software (Armonk, NY, USA). Shrimp Survival percentage and their significance levels were generated for turbidity treatments using Graph Pad software (Version 7.04 for Windows, San Diego California, USA). Immunological (THC, PO, and SOD) and stress metabolites (Glucose, lactate, and osmolality) collected from the experiment were subjected to ANOVA. Data were reported as mean ± standard deviation (SD) and the means of each individual group were compared by Duncan's test.

RESULTS

Exposure of *P. vannamei* to different levels of turbid water for three weeks resulted in significant changes in immune responses and stress metabolites with notable marks in gills of shrimp are discussed in detail.

Effect of Turbidity on Survival

T120 and T60 registered 20 and 10% mortality respectively compared to 5% in T30 and control. Mortality rates of shrimps exposed to different levels of turbidity for three weeks are given in Table 1. No mortality was observed in the first two weeks of experiment and mortality started on the 19th day of the experiment. Shrimp in T30, T60, and T120 showed 1.1, 1.9 and 4 times more risk of dying compared to control.

Effect of Turbidity on Immunological Response

A significant ($p < 0.05$) decrease in the THC ($x10^5$ cells ml^{-1}) count was observed from day-1 to day-14 in all the treatments (Figure 1A). Amongst the treatments, the highest THC was observed in shrimps exposed to T30 on day-1 (176.5±9.1) and decreased significantly to 153.5±7.7 by day-21. Among the treatments, a significant decrease in THC was observed with increase in turbidity levels and less hemocyte count recorded in T120 treatment by the end of the experiment (108.5±10.6).

PO Activity (U ml^{-1}) decreased significantly ($p < 0.05$) in all the treatment groups compared to control (Figure 1B). After 7days exposure to turbidity, shrimp showed reduced PO activity and it further reduced with exposure time and by day-21 shrimp showed least PO activity in T60 (0.3948±0.02) and T120 (0.3688±0.01). Statistical analysis revealed that treatment and exposure time had a significant impact on PO activity ($p < 0.05$). Among the treatment groups, the highest PO (0.5882±0.04) was recorded in T30 treatment and reduced significantly by day-21 (0.4498±0.06).

SOD activity of shrimps was significantly decreased in all treatments with respect to exposure time compared to control (Figure 1C). After exposure to turbidity for 24 h, SOD activity increased in all the treatments compared to control and significantly decreased by the end of the experiment. Among the treatments, the highest SOD was noted on Day-1 of the experiment in T30 (0.3093±0.01) and lowest activity was observed in T120 (0.2350±0.01) on day-21. SOD activity was stable over time for two weeks and reduced significantly in all the treatments by the end of the 3rd week. Treatment and time interaction did not impact SOD activity significantly.

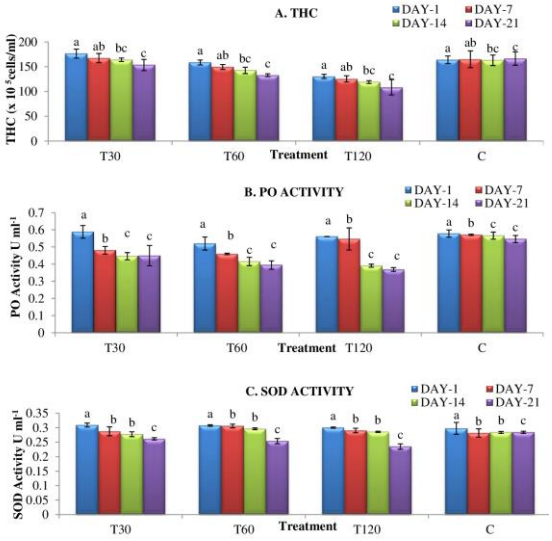

Figure 1. Effects of turbidity on immunological response in *P. vannamei*. A. THC-Total hemocyte count, B. Phenoloxidase (PO) Activity and C. Super Oxide Dismutase (SOD) Activity. Bars represent means±SD; Different alphabets indicate significant difference between time intervals within each turbidity level.

Effect of Turbidity on Stress Metabolites

There was no significant difference in glucose with respect to the exposure time. All the treatments showed significantly ($p < 0.05$) high activity compared to control (Figure 2A). Among the treatments, the highest value was recorded in T120 (85.2±0.071) on day-21 and lowest value on day-1 of T30 (75.2±0.078).

Hemolymph lactate (mg dl^{-1}) activity changed under turbidity treatments (Figure 2B). Lactate increased significantly ($p < 0.05$) with respect to the exposure time. Highest lactate activity recorded on day-21 in T120 (8.2±0.07) followed by T60 (8.0±0.06) and T30 (7.6±0.50). All the treatments showed high lactate activity by the end of the experiment compared to control. After 24 h of exposure, compared to control, lactate concentration decreased with increase in turbidity levels and recovered to a normal level after one week and again increased by the end of 3rd week. Statistical analysis proved that there was no significant interaction among the treatments, whereas treatment X time interaction had significant ($p < 0.05$) impact on lactate activity.

Variation was observed in hemolymph osmolality (mOsm Kg^{-1}) in all the turbidity treatments (Figure 2C). Among the treatments significant ($p < 0.05$) changes were observed and the

Table 1. *Results of Cox's regression for turbidity treatments using survival as an explanatory variable. Control treatment as a reference.*

Treatment	B	SE	Sig.	Exp (B)	95.0% CI for Exp (B)		Mortality (%)
					Lower	Upper	
Control							5±1.5
T30	-11.144	258.906	0.966	1.101	0.087	3.400	5±2.5
T60	0.662	1.225	0.589	1.939	0.176	21.382	10±2.0
T120	1.387	1.118	0.215	4.002	0.447	35.809	20±3.5

highest osmolality was recorded in T120 (961.7±148.6) followed by T30 (811.6±24.9) and T60 (646.8±162.2) on day-21. No significant differences were observed with respect to exposure time and interaction between treatment x time.

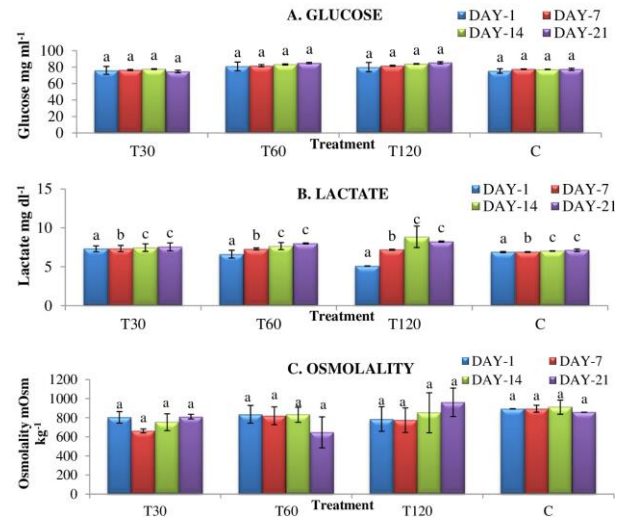

Figure 2. Effects of turbidity on *P. vannamei* on immunological response A. Glucose, B. Lactate and C. Osmolality. Bars represent means±SD; Different alphabets indicate significant difference between time intervals.

Effect of Turbidity on Gills

At low resolution, a normal arrangement of gill filaments and lamellae was observed in control gills (Figure 3A). SEM studies revealed significant and notable deposits in the shrimp gills after turbidity exposure. The variations in the surface architecture of the gill filaments and fused epithelial cells of secondary lamellae with turbid particles were observed in T120 (Figure 3D). In T30 and T60, at higher magnification of 10 µm, fine turbid particles (Figure 3B) and coagulated turbid particles (Figure 3C) were seen within the filaments. The gill color of the shrimp in control and T30 was dusky white, whereas T120 and T60 treatments, pale brown gills, and even deposits were clearly seen on the gills with the naked eye.

DISCUSSION

Penaeus vannamei grown in marsh-earth ponds might be adversely affected by suspended sediments. Studies over the past few decades have focused on the effects of turbidity on prey consumption and growth (Sweka and Hartman, 2001) and oxygen consumption (Alexander, Thorp, and Fell, 1994). However, this study highlights how turbidity at different levels affects *P. vannamei* immunological and stress response. This knowledge would clearly improve our understanding of how shrimp react to turbidity exposure. Exposure of *P. vannamei* to varying turbidity levels for three weeks caused a marked increase in the mortality rate of 20% and 10 % in T120 and T60 treatments, respectively. Similar mortality response has been observed in Rainbow trout exposed to 36 mg l^{-1} of kaolin inorganic clay i.e., 105 NTU (Goldes *et al.*, 1988) and the study on *M.japonicus* by Lin *et al*., (1992) also reported 5% mortality after a 3-week exposure to turbidity (65±15 NTU). In the present study, shrimp mortality was observed only on the 19th day of exposure indicating turbidity effects are chronic in nature, not leading to the immediate death of the animal.

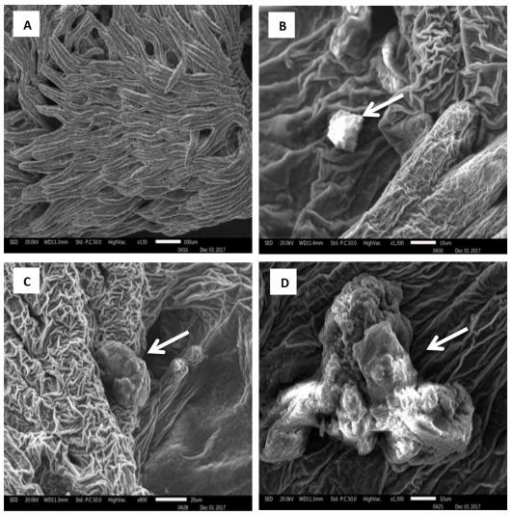

Figure 3. Scanning electron micrographs showing effects of turbidity (NTU) after 3week exposure on gills of *P. vannamei* A. Broad view of control shrimp gill filaments and lamellae showing normal morphological features. B. Magnified view of gills with deposits of turbid particles after exposure to 30 NTU. C. Magnified view of coagulated turbid particles after 3 week exposure to 60 NTU. D. Magnified view of gills with turbid particles fusion of secondary lamellae, epithelium in the interlamellar regions after exposure to 120 NTU.

Shrimp lack an adaptive immune system and rely on innate immune system cellular (hemocytes) and humoral events. Hemocytes play a major role in scavenging foreign particles by encapsulation, phagocytosis (Raman, Arumugam, and Mullainadhan, 2008) and PO that have been found to play several important roles in the defense reactions with formation of melanin (Sritunyalucksana and Söderhäll, 2000). SOD is one

of the important antioxidant enzymes to respond to stress (Campa-Córdova *et al*., 2002). Joseph and Philip (2007) observed a reduction of THC, PO and SOD activity in *P. vannamei* in response to environmental and physiological stress. Studies on *P. vannamei* revealed a decrease in THC, PO and SOD activity under ammonia stress (Liu and Chen, 2004), pH stress (Wang *et al*., 2009), salinity stress (Esparza-Leal *et al*., 2019), temperature stress (Jia *et al*., 2014) and this trend was also confirmed in the present work on *P. vannamei* under turbidity stress. The results of the present experiment clearly showed that increased levels of turbidity have a significant role on decrease in the immune response. Leadprathom (2012) reported an early response with sediment resuspension on phenoloxidase activity in penaeid shrimp postlarvae. Shaw and Richardson (2001) and Sutherland and Meyer (2007) revealed lower immune system response in rainbow trout with suspended fine particles.

In the present study with T60 treatment, the osmolality reduced by 24.6 times compared to control. This is in line with the results of Lin *et al*. (1992), on *Penaeus japonicus* exposure to 65±15 NTU. Lactate is the major end product in crustaceans under stress conditions (Hill, Taylor, and Strang, 1991). The elevated lactate levels not excreted must be slowly broken down through the process of gluconeogenesis, which requires more oxygen uptake for the removal of lactate (Hsieh *et al.,* 2008). The dramatic drop in oxygen consumption with increasing turbidity was reported in Zebra mussel *Dreissena polymorpha* (Alexander, Thorp, and Fell, 1994) and *Penaeus japonicus* (Lin *et al*., 1992). Hence, accumulated lactate in hemolymph creates osmotic pressure by intracellular metabolic acidosis (Kieffer, 2000) and increasing the osmolality. This is the possible reason for the elevated lactate and osmolality levels in T120 treatments. Servizi, Gordon, and Martens (1987) reported that suspended river sediments exposure to sockeye smolts *Oncorhynchus nerka* resulted in hypo-osmoregulatory capacity and upshot in glucose levels. Increased levels of glucose and osmoregulatory ability (Redding, Schreck, and Everest, 1987; Servizi and Martens 1992) were reported in coho salmon under increased suspended sediments. Hyperglycemia, in aquatic animals, is a typical sub lethal response to environmental stressors (Radford *et al*., 2005), supported the results of glucose activity in the present study. The present study is also corroborated with the report of US Fish and Wildlife Service (USFWS 1998), the suspended sediment stress response itself may compromise the organism's immune system thereby affecting mortality rates in bull trout. This clearly supports the present trails of T120 treatment with a compromised immune system, showing four times more risk of dying compared to control.

The SEM examination of gills in T120 treatment clearly shows the deposits of turbid particles with fused epithelial cells. A similar report was observed in dead shrimps after turbidity exposure (65 NTU) for three weeks; the gill chambers (in particular gill lamellae) became brown-gray and were densely covered with suspended particles (Lin *et al.*, 1992). Redding, Schreck, and Everest (1987) reported that gill tissue in yearling coho salmon and steelhead exposed to suspended topsoil between 1.7 and 2.7 g l^{-1} for 7 to 8 d was similar to that in the control fish. Penaeid shrimps still require regular water current from an external medium to flush the gill chamber (Dall *et al*., 1990). Turbidity exposed shrimp need to continuously clean their gill chambers from excessive turbid particles, which requires more energy and metabolic rate. It has been reported that coho salmon experienced gill flaring after suspended sediment exposure (Berg and Northcote, 1985) and exposed Arctic grayling *Thymallus arcticus* showed an increase in cough reflex (McLeay *et al*., 1987), appeared like to improve the cleaning efficacy. This may be the possible reason for the mortality of shrimp in T60 and T120 exposed animal due to the accumulated turbid particles, clogged the gill chamber and inefficient to clean the gills. This caused gill suffocation with the inadequate exchange of oxygen and ions from the external medium.

CONCLUSIONS

The effect of a turbidity stress response is dependent on synergistic factors such as duration of exposure, frequency, magnitude, temperature, and other environmental variables. Results from the present study indicate that turbidity in intensive vannamei ponds should be <30NTU and suggest that lactate and osmolality act as an indicator of stress under turbidity stress. The impact of turbidity appears to occur primarily in gills. Improved water quality and altered management may allow moderately affected gills to recover. The present study concludes that water turbidity can cause immunological and metabolic changes in *P. vannamei.* Shrimp mortality occurred after 18 days in 60 and 120 NTU, indicating the optimum turbidity as <30 NTU. High turbid particles in the rearing medium caused the gills blockage apparently as a compensating reaction to the disruption of osmotic and ionic balance.

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