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Investigation on the infectious nature of Running Mortality Syndrome (RMS) of farmed Pacific white leg shrimp, *Penaeus vannamei* in shrimp farms of India

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ABSTRACT

Shrimp aquaculture in India has made significant strides especially after the introduction of Pacific white shrimp in the year 2009. However, intensification of culture practice has exacerbated several disease issues. Apart from infectious diseases, the Indian shrimp aquaculture has been witnessing certain syndromes, affecting productivity. One such syndrome associated with significant morbidity and mortality is popularly termed as running mortality syndrome (RMS) by shrimp farmers. Since 2011, RMS has been widely prevalent in the shrimp farms in Andhra Pradesh (AP) and Tamil Nadu (TN). The affected shrimp show patches of whitish musculature in the abdominal segments as a clinical sign with continuous low-level mortalities, especially after about 35–40 days of shrimp culture. We tried to investigate if this kind of mortality is infectious in nature. Investigations conducted in 34 farms comprising 25 RMS affected and 9 healthy farms were tested negative for all the major OIE listed and other pathogens such as white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), hepatopancreatic parvo virus (HPV), infectious myonecrosis (IMNV), Taura syndrome virus (TSV), yellow head virus (YHV), and *Penaeus vannamei* noda virus (PvNV). Bacteriological examination of haemolymph and hepatopancreas of RMS affected shrimp showed the predominance of *Vibrio* spp., such as *Vibrio parahaemolyticus* and *Vibrio azureus*. Histopathological examination of the hepatopancreas was found to be largely normal, except for haemocytic infiltration in the abdominal segments. Hemolymph of affected shrimp was observed to have less concentration of major and trace minerals than healthy ones. Bioassay through feeding RMS affected shrimp tissue to healthy shrimp and co-habitation experiment of healthy shrimp with the affected animals failed to induce RMS. When maintained in water with optimal physico-chemical parameters, affected shrimps showed recovery within 6–7 days. On the other hand, environmental parameters of pond waters such as total ammonia nitrogen, nitrite nitrogen and turbidity were relatively higher than the optimal values in RMS affected farms. Multiple correspondence statistical analysis of critical factors indicated running mortality to be associated with high stocking densities, high nitrite-N, and high turbidity. Though the study could not identify any specific known aetiological agent associated with RMS affected shrimp, failure to reproduce the syndrome by bioassay, the recovery of affected shrimp under the optimal environmental conditions and the positive correlation with critical environmental parameters and the stocking densities to mortality rates clearly suggest RMS to be a pond ecosystem or pond management associated syndrome rather than infectious in nature and thus can be overcome through best management practices.

1. Introduction

Shrimp aquaculture has made significant progress over the last decade in India, with shrimp production reaching 487 thousand metric tons during 2015–16, which was just about 144 thousand tons during 2006–07 (MPEDA, 2016). This enhanced production could be largely attributed to the successful introduction of Pacific white shrimp, *Penaeus vannamei,* when India's brackishwater aquaculture sector was looking for alternate species, several positive attributes associated with this species made it as a preferred choice for shrimp farming (Davis et al., 2004). With the availability of specific pathogen free (SPF) broodstock through import and verification of the disease free status at

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the aquatic quarantine facility (AQF) ensured the availability of good quality seed in the country (Remany et al., 2010) and *P. vannamei* culture expanded rapidly, particularly in the two Indian maritime states, Andhra Pradesh and Tamil Nadu.

Over the last couple of decades, diseases such as white spot disease caused by white spot syndrome virus (WSSV), yellow head disease caused by yellow head virus (YHV) and Taura syndrome caused by Taura syndrome virus (TSV) have heavily impacted shrimp aquaculture in the Asiatic region and the Americas resulting in the collapse of the shrimp industry. The combined loss from shrimp diseases at the global level from 11 shrimp farming countries for the period 1987–1994, was estimated at US$3019 million (Israngkura and Sae-Hae, 2002). WSD alone is estimated to have caused losses of over US$6 billion since its emergence in 1992 (Lightner et al., 2012; World Bank, 2014). Since 2009, the early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) has caused huge economic losses to shrimp farming in China, Vietnam, Malaysia and Thailand. Shrimp production in the Southeast Asian region dropped to about 60% and loss due to AHPND was reported to be in excess of US$1 billion per year (FAO, 2013). AHPND was also detected in Mexico in 2013 with > 80% mortality in about 50% of its shrimp farms in operation and the loss was estimated to be US$116.2 million.

The Pacific white shrimp was introduced for brackishwater aquaculture in India in the year 2009 and the private sector embraced the new species with great interest. However, soon it was realised that the SPF status did not protect this species being affected by the existing viral pathogens such as WSSV (Balakrishnan et al., 2011; Otta et al., 2014). Disease issues continued to confront the shrimp farming sector, affecting productions. Since 2011, *P. vannamei* farms in India were encountering low and continuous mortalities. This condition results a small percent shrimp mortality in the affected pond on daily basis. As the mortality continues on daily basis till the rest of the culture period, it is called as “Running Mortality Syndrome (RMS)”. Usually the RMS started after 35–40 days of culture (DOC) with low mortalities and as the culture progressed, the mortality rate also increased and the problem becoming acute at around 90 DOC and the farmers were forced to prematurely harvest the crops. The development of white patches in the junctions of 2nd to 4th segments was the only observable clinical sign in RMS. This clinical sign of RMS was different from that described for infectious myonecrosis virus (IMNV) (Poulos et al., 2006) or *Penaeus vannamei* noda virus (PvNV) (Tang et al., 2007). In RMS, focal to extensive white necrotic areas in striated muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened usually observed in infectious myonecrosis could not be observed. Though RMS clinical sign was somewhat similar to that described by Melena et al. (2012), it was wide spread in the latter case.

The main objective of this study was to understand the infectious nature of this chronic mortality (running mortalities) of farmed Pacific white legged shrimp carried out during 2013–2014 in the states of Andhra Pradesh (AP) and Tamil Nadu (TN) in the southeast coast of India based of the information on farming, microbiological, histopathlogical and molecular diagnostic investigations. Additionally, pond environmental parameters were also analysed randomly to obtain any clue regarding its association with such parameters.

2. Material and methods

2.1. Information on the farms

Water, soil and shrimp samples were collected from a total of 34 farms comprising 16 from Nellore and 11 from West Godavari districts in Andhra Pradesh and seven from Nagapattinam district of Tamil Nadu. Out of the 34 shrimp farms, nine farms were healthy and the remaining farms were affected with running mortality syndrome (RMS) or continuous low mortalities. Samples were collected during three stages; one during the early stage (between 35 and 45 DOC when mortality starts), one in the middle phase (during 60 to 75 DOC) and one during the time of harvest (90 to 120 DOC). Since we were interested in finding out the infectious agent associated with the disease, more number of samples from different farms and different locations were collected rather than frequent samples from few farms/locations. General information including stocking density, pond area, source water and health condition of shrimp was obtained from all the farms. Details of mortality, onset of mortalities, its pattern and progress and loss in production were collected from the affected ponds.

2.2. PCR analysis for viral pathogens

Gills, hepatopancreas, intestine and pleopods from healthy or moribund shrimps with and without typical clinical signs were dissected out and collected separately in 90% ethyl alcohol and RNA later for the analysis of OIE listed DNA and RNA viruses. Another set of samples were stored in ice and transferred to the laboratory for further analysis. DNA and RNA was extracted from the respective tissue samples and subjected to PCR and agarose gel electrophoresis for the detection of WSSV, IHHNV (infectious hypodermal and hematopoietic necrosis virus), YHV (yellow head virus), IMNV (infectious myonecrosis virus) and TSV (Taura syndrome virus) as described earlier (OIE, 2014; Otta et al., 2014). The detection of MBV (monodon baculo virus) was carried out as per protocol and described by Belcher and Young (1998). PvNv (*Penaeus vannamei* noda-virus) was tested using the method of Tang et al. (2007).

2.3. Bacteriological analysis

Haemolymph samples collected with a sterile syringe directly from the heart and the hepatopancreas (HP) samples were inoculated on to thiosulphate citrate bile salts agar (TCBS) and Zobell's marine agar (ZMA) plates. Animals with typical clinical signs, the parts were washed in sterile phosphate buffer saline (PBS), swabbed and inoculated on to thiosulphate citrate bile salts agar (TCBS) and Zobell's marine agar (ZMA) plates. Intestine and stomach of shrimp were inoculated into tubes containing 2 ml sterile tryptose soya broth (TSB) with 1.5% NaCl. All the inoculated plates and tubes were transported to the laboratory and incubated at 30 °C in an incubator. After overnight incubation, the plates were examined for the development of bacterial colonies. From the TSB broth culture tubes, TCBS and ZMA plates were inoculated to obtain pure cultures.

Morphologically distinct representative colonies from the culture plates were further purified by streaking on fresh ZMA plates. The pure colonies were grown in TSB containing 1.5% NaCl overnight and subjected to phenotypic identification following biochemical key for the identification of all recognized *Vibrio* spp. (Noguerola and Blanch, 2008). DNA was extracted from the overnight culture by the CTAB method as described by Wilson (1994). The DNA was subjected to PCR using primer pairs targeting the 16S rDNA gene as previously described (Weisburg et al., 1991). The amplified PCR products were purified using a gel extraction kit following manufacturer's protocol (Qiagen, USA). These purified PCR products were sequenced and the bacteria were identified by comparing DNA sequence data in the GenBank database using the BLAST program available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

The DNA was extracted from the *Vibrio parahaemolyticus* isolates and PCR was carried out with metalloprotease gene specific primer to further confirm the identity of *V. parahaemolyticus* (Luan et al., 2007). The *V. parahaemolyticus* isolates were then tested by AP3 PCR protocol to detect Tox A protein gene to detect if these isolates belonged to AHPND (VP_{AHPND}) strains as described earlier (Sirikharin et al., 2014). Additionally, the shrimp tissues such as hepatopancreas and stomach were enriched by incubation in TSB supplemented with 1.5% NaCl for 4 h at room temperature and subjected to PCR to detect Tox A protein gene by AP3 protocol. All these samples were subsequently also verified

by the AP4 protocol for the presence of AHPND bacteria (Dangtip et al., 2015).

2.4. Histopathology

Live and moribund shrimps were collected and fixed immediately by injecting Davidson's fixative into the hepatopancreas and abdomen region. After 24 h, the fixed samples were transferred to 70% alcohol for storage. The hepatopancreas was dissected out and processed for histology as described by Bell and Lightner (1988). The haematoxyline and eosin (H & E) stained tissue sections were observed under a microscope (Leica, USA) and photographed.

2.5. Bioassay with affected animals

2.5.1. Test for recovery

Animals showing typical clinical signs of RMS from different farms were collected and brought to the laboratory and 7–9 animals from different farms were kept in a 100 l FRP tank with filtered sea water under aeration. The salinity of water was 22 ppt and temperature 28 °C. The animals were fed twice daily at 5% of their body weight. About 30% of tank water was replaced daily and the animals were observed twice in a day for any mortality or abnormality.

2.5.2. Oral transmission of RMS

The transmission of RMS to healthy pathogen free juvenile *P.vannamei* shrimps weighing ~8–10 g through oral route was examined by feeding experiments. WSSV, IHHNV and MBV free status of these shrimp was ensured by PCR protocols as described earlier. The animals were distributed into three groups containing five shrimp each in triplicate in 100 l FRP tanks and acclimatized for 72 h and fed with commercial pellet feed. Shrimp with typical clinical signs of RMS were collected from the farms and used for experimental transmission by feeding. The shrimp were fed twice with the freshly homogenised meat of RMS affected shrimp at the rate of 5% of body weight. Thereafter, the shrimp were fed with CIBA grow-out pellet feed. A group of shrimp fed only with CIBA grow-out pellet feed was maintained as control. The shrimps were examined twice daily for any mortality or abnormality.

2.5.3. Transmission of RMS by co-habitation

The infective nature of RMS, if any, was examined by co-habitation of affected animals with healthy animals. Five virus free healthy juvenile *P. vannamei* shrimps weighing ~8–10 g were maintained along with equal number of animals with clinical signs of RMS, separated by nylon net in 500 L FRP tanks. These animals were maintained under optimum water quality parameters and aeration. The animals were fed with commercial pellet feed. The animals were observed twice daily for any abnormalities or clinical signs of infection.

2.6. Collection and analysis of water, sediment and shrimp hemolymph samples

2.6.1. Onsite measurements

Samples were collected only once from a single pond/location. However, care was taken to represent the running mortality status, that is samples were representatives of all the stages of the condition that is otherwise prevailed in a single pond. Shrimp pond water quality parameters such as pH, salinity, turbidity and soil redox potential were measured immediately at the sampling site using multi-parameter water quality analyzer (DS5, Hach, USA). Electrodes were calibrated prior to analysis following manufacturer's instructions. The parameters measured onsite were stored and viewed in the surveyor provided by the manufacturer and recorded.

2.6.2. Collection of water, soil and shrimp hemolymph samples

Water samples from the culture ponds were collected in clean 1 l plastic bottles. Soil samples from the culture ponds were collected from the sediment-water interface from corners and centre of ponds, pooled and stored in air tight polythene bags and transported to the laboratory in ice for further analysis. Hemolymph was drawn from ventral sinuses of shrimps from each of the culture ponds separately using 1 ml sterile syringe containing anticoagulant (0.45 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate,10 mM EDTA, pH 4.6) and transported to the laboratory in dry ice and stored at −80 °C for mineral analysis.

2.6.3. Analysis of water and soil parameters

Calcium, magnesium, total hardness, alkalinity, total ammonia nitrogen (TAN), nitrite-N and sulfide analysis was carried out as per the standard methods (APHA, 2012; AOAC, 1997). Soil samples were air dried and ground to fine powder with mortar and pestle, passed initially through 2 mm sieve and stored in air tight plastic bags until further analysis. Some portion of the soils was again passed through 0.2 mm sieve and stored for the analysis of organic carbon (Jackson, 1973). pH and electrical conductivity (EC) were measured using pH and EC meter (ELICO, India). Organic carbon content in soil was analysed by Walkley and Black (1934) method.

2.6.4. Analysis of minerals in shrimp hemolymph

Shrimp haemolymph minerals were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent 5100) after digestion (Microwave PRO, Anton Par) using nitric acid and hydrochloric acid (Jannathulla et al., 2017). Briefly, 300 µl of hemolymph was transferred to inert polymeric microwave vessels and to this, 6 ml of HNO_3 and 2 ml of H_2O_2 were added, sealed carefully and transferred to microwave digestion system. Digestion was performed with the temperature setting of 180 °C for 10 min, 70 °C for 8 min and additional 10 min for venting. Digested samples were allowed to cool and made upto a final volume of 10 ml with distilled water. The calibration curve was plotted and checked for linearity at five different concentrations of 2, 4, 6, 8 and 10 mg l^{-1} with 23 element standard mix (Merck, Cat No: 1.11355.0100). The analytical conditions were maintained at 0.6 l min^{-1} nebulizer flow, 0.2 l min^{-1} auxiliary flow and 15 l min^{-1} plasma flow.

2.7. Statistical analysis

The significance between RMS affected and healthy ponds with respect to water and soil parameters and content of minerals in the hemolymph was compared with student's 't' test. A tool of categorical data analysis i.e., Multiple Correspondence Analysis (MCA) was used to find out the association between the categories of critical variables and shrimp mortality. The statistical analysis was carried out with SAS package.

3. Results

3.1. Farming information

The study area included Nellore and West Godavari districts in AP and Nagapattinam district in TN (Fig.1), being major shrimp farming hubs and reporting frequent episodes of RMS. Among the 34 farms, nine farms were healthy and 25 farms were affected by varying degrees of mortality. RMS affected farms undertaken for this study had wide variation in terms of source water, stocking density and days of culture (DOC) (Table 1). In one of the RMS affected farms, shrimp mortalities started as early as 35 DOC and continued up to 120 DOC in some other farms. The stocking density in these farms ranged from 24 m^{-2} to 87 m^{-2}. The stocking density in healthy ponds ranged from 24 to 45 m^{-2}, whereas it was 44 to 87 m^{-2} in RMS affected ponds. The source of water for the farms was either creek (n = 14), bore well (n = 10) or a mixture of both (n = 10) in Nellore and West Godavari

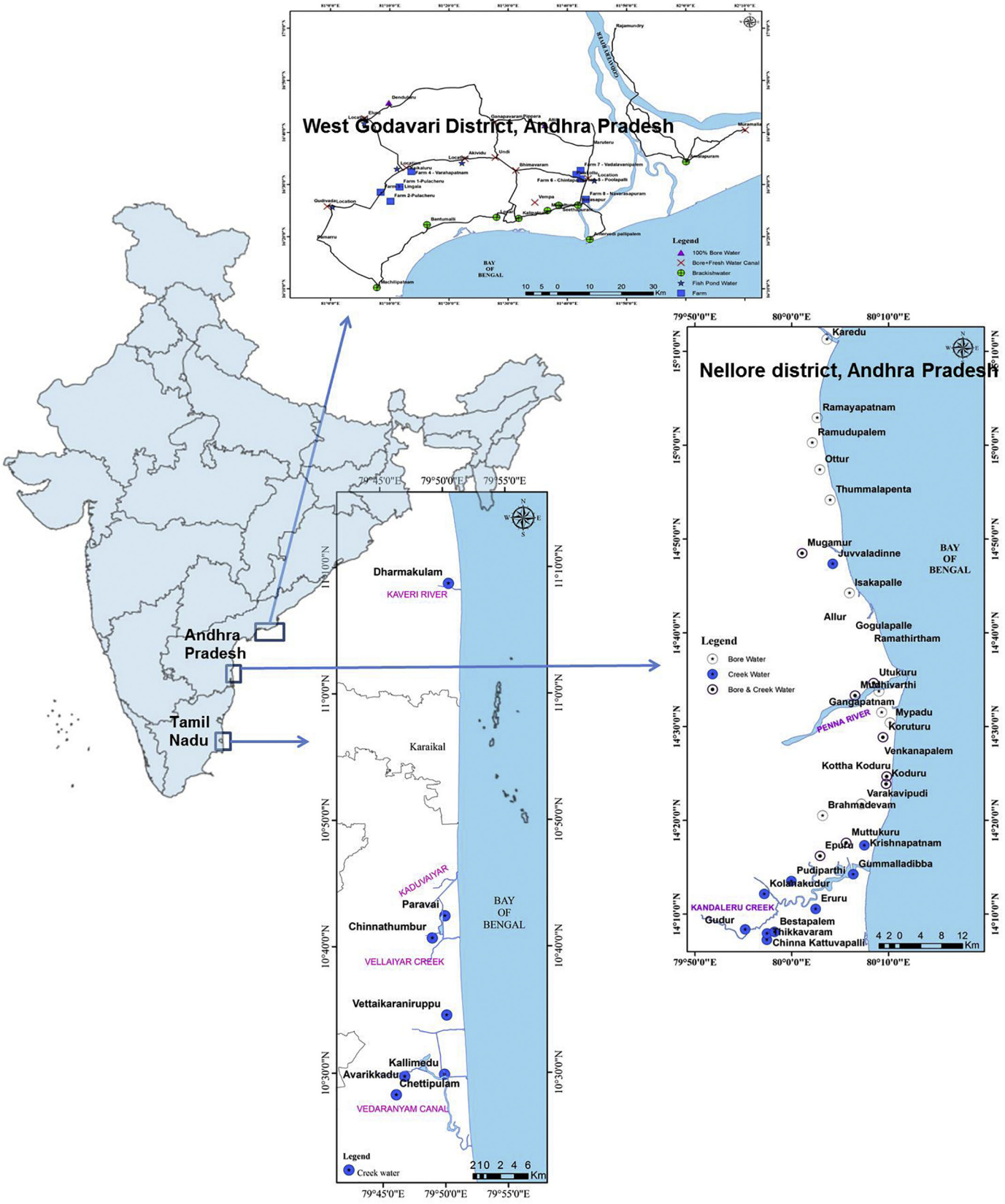

Fig. 1. Location of farms investigated for RMS in Andhra Pradesh and Tamil Nadu, India.

Table 1
Location, water source and shrimp survival in normal and RMS affected farms.

District (state)	Source water	Status		Mortality during DOC	Survival (%) at harvest in RMS affected ponds
		Healthy (SD)	Affected (SD)		
West Godavari (AP) (*n* = 11)	Bore well (*n* = 7)	3 (24–30)	4 (44–49)	48–85	32–46
	Creek (*n* = 2)	1 (24)	1 (57)	67–84	48
	Mixture of BW and creek (n = 2)	1 (41)	1 (46)	35–72	45
Nellore (AP) (*n* = 16)	Bore Well (*n* = 3)	0	3 (47–70)	64–102	30–38
	Creek (*n* = 5)	2 (40–50)	3 (62)	64–120	42–50
	Mixture of BW and creek (*n* = 8)	1 (40)	7 (47–87)	61–116	38–46
Nagapattinam (TN) (n = 7)	Creek (*n* = 7)	4 (28–45)	3 (45–60)	50–98	35–60

SD- shrimp stocking density m^{-2}; AP: Andhra Pradesh; TN: Tamil Nadu; DOC: Days of culture.

Fig. 2. Typical clinical signs of RMS affected shrimp (white patches (arrow) between 2nd and 4th segments).

districts, whereas the farms in Nagapattinam district had only creek as the source water. RMS was observed in the 25 farms irrespective of source water.

3.2. Clinical signs

The onset of the RMS was found to occur between 35 and 45 days of culture (DOC), where a small portion of shrimp population suffered mortality and the farmers could collect these dead shrimps by netting. The shrimps suffering from this problem develop white patches between the 3rd and 4th abdominal segment (Fig. 2). As the problem advances, animals show red discoloration of the body, stop feeding and subsequently die. The mortality percentage then gradually increases as the days of culture progresses. The mortality becomes severe at around 90 DOC and farmers were forced to harvest their crop prematurely. On an average, about 40% of the stocked shrimp survive and grow to a harvestable size. RMS often caused pond biomass to fall substantially in farms with mortality rates reaching 70% (Table 1).

3.3. Detection of viral pathogens

All the shrimp tissue samples tested were negative for the OIE listed viral pathogens (WSSV, IHHNV, TSV, IMNV and YHV) and also three other commonly prevalent viral pathogens (MBV, HPV and PvNv) of penaeid shrimp (Data not shown), suggesting that RMS may not be due to any of these viral pathogens.

3.4. Bacteriological analysis

Out of the 34 farm samples processed, HP samples from 15 farms did not produce any growth on TCBS medium. Phenotypic identification of the 36 bacterial isolates from 19 farms indicated that these isolates were predominantly *Vibrio* species. *V. parahaemolyticus* was isolated from shrimp hepatopancreas samples of five farms, *V. proteolyticus* and *V.coralliilyticus* from four farm samples each and *V.alginolyticus* from three farm samples. Swabs taken from the shrimp abdomen showing clinical signs did not show any bacterial growth.

Table 2
Bacteria isolated from gut / hepatopancreas of shrimp affected with chronic mortalities.

Location of shrimp farms	Farm	Bacteria isolated on TCBS (phenotypically identified)	Predominant bacteria
West Godavari	WG1	No growth	–
	WG2	*V.parahaemolyticus, V. proteolyticus*	*V.parahaemolyticus*[a]
	WG3	*V. proteolyticus, V.parahaemolyticus*	*V.proteolyticus*
	WG4	No growth	–
	WG5	No growth	–
	WG6	*V.coralliilyticus, Vibrio azureus, V.nereis*	*V.coralliilyticus*
	WG7	*V.alginolyticus, V. proteolyticus, V.parahaemolyticus,*	*V.alginolyticus*
	WG8	*Psedomonas sp., V.alginolyticus*	*V.alginolyticus*
	WG9	*V.mimicus, V.alginolyticus*	*V.alginolyticus*
	WG10	No growth	–
	WG11	No growth	–
Nellore	NE1	No growth	–
	NE2	No growth	–
	NE3	*V.proteolyticus, V.coralliilyticus, V.alginolyticus,*	*V.proteolyticus*
	NE4	No growth	–
	NE5	*V.parahaemolyticus*	*V.parahaemolyticus*[a]
	NE6	*V.vulnificus, V.proteolyticus*	*V.proteolyticus*[a]
	NE7	No growth	–
	NE8	No growth	–
	NE9	*Vibrio proteolyticus*	*Vibrio proteolyticus*
	NE10	No growth	–
	NE11	No growth	–
	NE12	*V.coralliilyticus, Vibrio azureus*	*V.coralliilyticus*
	NE13	No growth	–
	NE14	*V.alginolyticus*	*V. natreigens*[a]
	NE15	No growth	–
	NE16	No growth	–
Nagapattinam	NG1	*V.parahaemolyticus*	*V.parahaemolyticus*[a]
	NG2	*V.parahaemolyticus*	*V.parahaemolyticus*
	NG3	*V.alginolyticus V.parahaemolyticus,*	*V.parahaemolyticus*
	NG4	*V.azureus, V.coralliilyticus, V.parahaemolyticus*	*Vibrio azureus*[a]
	NG5	*V.fischeri, V.coralliilyticus*	*Photobacterium damselae*[a]
	NG6	*V.coralliilyticus,*	*V.coralliilyticus*
	NG7	*V.coralliilyticus, V.vulnificus*	*V.coralliilyticus*

[a] Confirmed by sequencing 16 s rRNA gene.

Predominant isolates phenotypically confirmed as *Vibrio* species were further confirmed by the 16 s rRNA gene sequencing (Table 2). All the isolates identified as *V. parahaemolyticus* either phenotypic methods or through 16 s rRNA gene sequencing were further verified by PCR

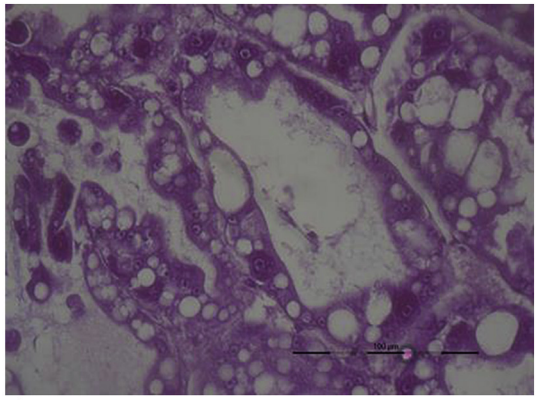

Fig. 3. Histology of RMS affected shrimp hepatopancreas (A) and muscle showing haemocytic infiltration.

amplification of VPM gene specific for *V. parahaemolyticus.* The identity of *V. parahaemolyticus* was confirmed by amplification of a 675 bp fragment of metalloprotease gene specific for *V. parahaemolyticus* (Fig. 3). All the *V. parahaemolyticus* isolates and the TSB cultures from stomach and intestine subjected to AP-3 PCR protocol could not amplify the 336 bp AHPND target and also did not amplify any of the two products (nested PCR) by AP4 primers, confirming that these *V. parahaemolyticus* isolates did not belong to AHPND strain. The stomach and intestine of RMS affected animals were also found to be AHPND negative by PCR (Data not shown).

3.5. Histopathological studies

Histological sections of hepatopancreas of RMS affected shrimp from most farms were normal, while some samples showed karyomegaly and increased inter hepatopancreatic tubular space with some haemocytic infiltration. The hepatopancreatic tubules appeared intact in most cases. In some cases, the B-cells showed vacuolation and the lumen contained a secretory granular material. The sections of these samples did not show occlusion bodies suggestive of any viral infection or cell necrosis or sloughing into the lumen as observed in the case of AHPND (Fig.4). Sections from muscle showed necrosis associated with haemocytic infiltration (Fig.4). Sections of gills were also largely normal.

3.6. Transmissibility of RMS

The bioassay experiments were conducted to understand the infectious nature of the RMS. After five days of tissues from the RMS affected shrimp were fed to the healthy shrimp, the challenged animals did not show any clinical signs of RMS. There were a few occasional moralities similar to that of the controls (Table 3). The co-habitation of the healthy animals along with affected animals did not result in manifestation of clinical symptoms of RMS or shrimp mortality. However, the affected animals showed recovery indicated by progressive disappearance of the white patches in the abdominal segments.

Further, the affected shrimp from different farms when maintained in wet lab facility with optimum water parameters did not show any mortality during the rearing period of 10 days (Table 4). Moreover, the animals got completely recovered from the clinical signs during the maintenance in the wet laboratory. All the recovered animals (based on the clinical sign) were found healthy and active. The recovery of RMS affected shrimp while rearing in sea water with optimal water parameters further suggests that the RMS might not be due to any infectious agent.

3.7. Source water quality

The pH of the creek water and bore well waters in all the three places did not differ significantly. However, there was a significant variation in the salinity of both bore water and creek water within and between the study areas. The salinity of bore well waters ranged from 7 to 38 ppt and creek water from 1.6–21 ppt. The salinity of the bore well water feeding in farms in Nellore district was found to be higher than the bore well water being used in farms in West Godavari district. Similarly the salinity of creek water also varied considerably within and between the study areas (2 to 5 ppt in West Godavari, 11 to 21 ppt in Nellore and 4 to 12 ppt in Nagapattinam). The turbidity of the creek water was higher and calcium, magnesium and total hardness levels were lower than the bore well water in all the districts. The water quality variables of source waters were well within the acceptable limits except for the total ammonia nitrogen (TAN) which was found to be higher than the optimum levels (Table 5).

3.8. Water and soil quality of healthy and RMS affected farms

Among the pond water quality parameters examined, turbidity and TAN were significantly higher in the RMS affected ponds (Table 6). All

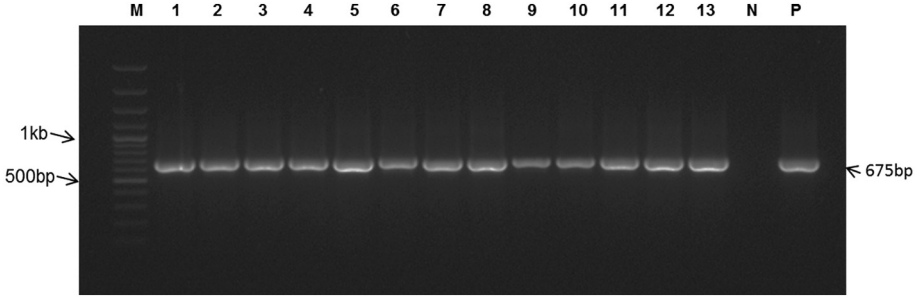

Fig. 4. Confirmation of *V.parahaemolyticus* isolates based on the presence of metalloprotease gene (vpM): lanes 1to13, N-Negative control, P-Positive control M-Marker (100 bp).

Table 3
Transmissibility of chronic mortality syndrome by bioassay and cohabitation experiments.

Experiment no	Nature of tissue used for bioassay	No. of healthy animals in each tank (triplicate)	Percentage mortality within 5 days	Co-habitation study (Healthy animal + affected animal (triplicate)	Percentage mortality /clinical signs within 5 days
1	Freshly dead animal with clinical sign	5	Nil	5 + 5	Nil
2	Frozen tissue (−80 °C) of dead animal with clinical sign	5	Nil	–	–
3	Freshly dead animal with clinical sign	5	20	5 + 5	Nil
4	Freshly dead animal with clinical sign	5	Nil	5 + 5	Nil
5	Freshly dead animal with clinical sign	5	20	5 + 5	Nil
6	Freshly dead animal with clinical sign	5	Nil	5 + 5	Nil

Table 4
Recovery of running mortality syndrome affected shrimp in wet laboratory.

Experiment no	Source of animals: animals with clinical signs from RMS affected ponds	No. of animals in each tank (triplicate)	Percentage mortality in 10 days
1	(Farm −1)	8	Nil
2	(Farm −2)	9	Nil
3	(Farm −3)	8	Nil
4	(Farm −4)	7	Nil
5	(Farm −5)	8	Nil

the RMS affected ponds had higher TAN mean value (0.73 to 1.26 ppm) than the permissible levels. Significant differences in nitrite and sulfide values were also observed among healthy and affected ponds, whereas no difference was observed in total alkalinity and total hardness. RMS was found to occur even in low saline ponds in West Godavari district, and hence salinity may not be playing any role on the occurrence of RMS.

With regard to sediment quality parameters, the average values of organic carbon and redox potential was 0.33 & 0.57% and −97 & −110 mV in healthy and RMS affected ponds respectively (Table 6). No significant difference was observed in organic carbon values between healthy and affected ponds. Higher organic carbon (0.6 and 0.57%) was recorded in healthy and affected ponds of Nellore farms. No significant difference was found in redox potential in healthy and affected ponds. All the ponds had reducing conditions as indicated by the redox potential. The pond soils of Nagapattinam district were more saline as indicated by electrical conductivity (8.61 to 9.91 dS m^{-1}) compared to West Godavari (1.66 to 2.99 dS m^{-1}) and Nellore (6.02 to 7.25 dS m^{-1}) and did not differ significantly between healthy and RMS affected ponds.

3.9. Minerals in shrimp hemolymph

Composition of minerals in the healthy and affected animals hemolymph samples in the three sampling districts was categorized based on the salinity (5–10, 10–20, 20–30 and 30–40 ppt) and source water (creek, bore well and mixture of both). In general there was a decreasing trend of all major minerals (Ca, Na, K and Mg) in RMS affected animals compared to healthy animals except in 5–10 ppt salinity range for Ca, K and Mg (Fig. 5A) and Na (Fig. 5B). Similar trend was observed for trace minerals (Zn, Fe Cu, and Se) at all salinity groups except for Cu (Fig. 6A) and Se (Fig. 6B) in 5–10 ppt group. The average values of Ca, Na, K and Mg were 537, 3774, 280 & 94 ppm and 507, 3650, 256 and 77 ppm for healthy and affected animals, over different salinity ranges respectively. Similarly, the average values of Zn, Fe, Cu and Se were 24, 0.416, 209 and 0.55 ppm and 20, 0.369, 188 and 0.478 ppm in healthy and affected animals, in 5–10, 10–20, 20–30 and 30–40 ppt salinity range, respectively. Significant difference ($p \leq .05$) was observed in the composition of Na, Ca, K, Mg, Zn and Fe and no significant difference ($p \geq .05$) for Se and Cu between the healthy and RMS affected shrimp.

With regard to source waters, no particular trend was observed between healthy and affected animals with respect to major minerals (Fig.5 A & B and Fig. 6 A & B). However, there was a decrease in trace minerals in affected shrimp compared to healthy animals irrespective of source water, except for Fe which was high in affected shrimp (0.48, 0.56 and 0.59 ppm) compared to 0.42, 0.3 and 0.58 ppm in healthy shrimp with creek, bore well and mixture of both cultured farms, respectively.

3.10. Critical parameters contributing to shrimp mortality

It is clear that mortality of shrimp is not because of single stressor (critical factor), but appears to be a combination of many stressors. Many a times, though ammonia and sulfide exceed the optimum level,

Table 5
Physico-chemical characteristics of source water in three shrimp farming areas.

Water quality parameters	West Godavari		Nellore		Nagapattinam
	BW	CW	BW	CW	CW
pH	7.23 ± 0.22	7.5 ± 0.32	7.44 ± 0.28	7.97 ± 0.41	7.61 ± 0.35
Salinity (ppt)	8.6 ± 1.5	3 ± 1.4	28 ± 10	16 ± 5.3	8 ± 4
Turbidity (NTU)	11.5 ± 9	61 ± 10	34 ± 23	50 ± 46	49 ± 29
TAN (ppm)	0.072 ± 0.028	0.458 ± 0.14	0.412 ± 0.327	0.321 ± 0.147	0.180 ± 0.03
Nitrite (ppm)	0.019 ± 0.006	0.043 ± 0.018	0.04 ± 0.02	0.076 ± 0.058	0.013 ± 0.002
Ca (ppm)	426 ± 232	42 ± 5.6	443 ± 96	338 ± 144	269 ± 222
Mg (ppm)	387 ± 134	64 ± 57	1297 ± 456	910 ± 285	305 ± 192
Total hardness (ppm)	2681 ± 1030	375 ± 245	6513 ± 1840	4640 ± 1450	1943 ± 1354
TA (ppm)	357 ± 127	187 ± 10	215 ± 16	170 ± 63	172 ± 27

BW- Bore well; CW- Creek water; TAN: total ammonia nitrogen.

Table 6
Physico-chemical characteristics of water and soil from healthy and running mortality syndrome affected shrimp farms.

	West Godavari (n = 11)		Nellore (n = 16)		Nagapatinam (n = 7)	
	Healthy	Affected	Healthy	Affected	Healthy	Affected
Water quality parameters						
pH	$7.22^{a} \pm 0.26$ (7.01–7.65)	$7.22^{a} \pm 0.23$ (6.85–7.53)	$7.33^{a} \pm 0.16$ (7.18–7.5)	$7.25^{a} \pm 0.18$ (7.03 ± 7.48)	$7.61^{a} \pm 0.36$ (7.07–8.32)	$7.79^{a} \pm 0.14$ (7.64–7.92)
Salinity (ppt)	$8^{a} \pm 1.87$ (5–10)	$6^{a} \pm 0.81$ (5–7)	$33^{a} \pm 3.1$ (30–36)	$35^{a} \pm 7.7$ (30–47)	$29^{a} \pm 8.3$ (15–37)	$21^{a} \pm 2.5$ (19–24)
Turbidity (NTU)	$83^{a} \pm 75$ (40–236)	$216^{b} \pm 178$ (127–535)	$280^{a} \pm 257$ (83–572)	$399^{b} \pm 507$ (41–1136)	$101^{a} \pm 70$ (54–183)	$202^{b} \pm 156$ (61–530)
TAN (ppm)	$0.851^{a} \pm 0.416$ (0.355–1.403)	$1.305^{b} \pm 0.724$ (0.701 ± 2.263)	$0.539^{a} \pm 0.379$ (0.195–0.947)	$0.866^{b} \pm 0749$ (0.143–1.83)	$0.798^{a} \pm 0.55$ (0.174–1.227)	$1.616^{b} \pm 0.987$ (0.331–3.385
Nitrite (ppm)	$0.035^{a} \pm 0.314$ (0.017–0.641)	$0.173^{b} \pm 0.247$ (0.033–0.608)	$0.028^{a} \pm 0.011$ (0.016–0.038)	$0.399^{b} \pm 0.33$ (0.029–0.683)	$0.135^{b} \pm 0.13$ (0.002–0.278)	$0.266^{a} \pm 0.288$ (0.039–0.697)
Sulfide-S (ppm)	0.077^{a}- 0.04 (0.022–0.141)	$0.153^{b} \pm 0.06$ (0.08–0.241)	$0.158^{a} \pm 0.064$ (0.113–0.231)	$0.174^{a} \pm 0.065$ (0.121 ± 0.268)	$0.08^{a} \pm 0.042$ (0.028–0.172)	$0.09^{a} \pm 0.056$ (0.0034–0.146)
Ca (ppm)	$255^{a} \pm 156$ (72–435)	$209^{a} \pm 69$ (76–278)	$612^{a} \pm 51$ (582–672)	$716^{a} \pm 96$ (582–806)	$423^{a} \pm 125$ (144–528)	$358^{a} \pm 66$ (288–397)
Mg (ppm)	$351^{a} \pm 164$ (97–503)	$236^{a} \pm 67$ (153–298)	$1281^{a} \pm 15$ (12,630 1290)	$1424^{a} \pm 341$ (1236–1935)	$1182^{a} \pm 571$ (131–1782)	$1034^{a} \pm 113$ (927–1153)
Total hardness (ppm)	$2103^{a} \pm 950$ (582–3102)	$1510^{a} \pm 422$ (829–1904)	$6869^{a} \pm 171$ (6720–7050)	$7728^{a} \pm 1591$ (6608–10,080)	$5985^{a} \pm 2518$ (1747–8736)	$5205^{a} \pm 498$ (4859–5777)
Total Alkalinity (ppm)	$226^{a} \pm 97$ (102–364)	$212^{a} \pm 93$ (102–335)	$213^{a} \pm 46$ (165–257)	$240^{a} \pm 45$ (189–291)	$200^{a} \pm 63$ (94–323)	$227^{a} \pm 69$ (149–283)
Soil quality parameters						
pH	$7.49^{a} \pm 0.28$ (7.15–7.28)	$7.59^{a} \pm 0.19$ (7.32–7.85)	$8.23^{a} \pm 0.13$ (8.12–8.39)	$8.18^{a} \pm 0.12$ (8.02–8.33)	$8.28^{a} \pm 0.28$ (8.06–8.6)	$8.32^{a} \pm 0.19$ (7.99–8.58)
EC (dS m^{-1})	$2.99^{a} \pm 1.86$ (1.38–5.68)	$1.66^{a} \pm 0.44$ (0.92–2.2.7)	$7.25^{a} \pm 2.75$ (5.67–10.4)	$6.02^{a} \pm 1.67$ (8.02–8.33)	$8.61^{a} \pm 5.12$ (2.77–12)	$9.91^{a} \pm 7.64$ (3.13–24)
OC (%)	$0.15^{a} \pm 0.07$ (0.09–0.24)	$0.15^{a} \pm 0.05$ (0.06–0.21)	$0.21^{a} \pm 0.133$ (0.123–0.368)	$0.26^{a} \pm 0.05$ (0.18–0.34)	$0.64^{a} \pm 0.28$ (0.38–0.94)	$0.57^{a} \pm 0.33$ (0.18–1.2)
Redox (mV)	$-81^{a} \pm 16$ (−188 to 44)	$-97^{a} \pm 126$ (−135 to 47)	$-101^{a} \pm 32$ (−105 to −68)	$-116^{a} \pm 21$ (−93 to −146)	$-110^{a} \pm 41$ (−62 to −135)	$-116^{a} \pm 74$ (−249 to −4)

Mean values of parameters in healthy and affected ponds in each district with different superscript letters are significantly different ($P \leq .05$).

Fig. 5. Concentration of major minerals in hemolymph of healthy and RMS affected shrimp cultured in varying salinities and different source waters: A. Calcium, Potassium & Magnesium and, B. Sodium.

mortality may not occur, but combinations of these factors are likely to make the animals stressed and subsequently may result in mortality due to possible secondary infections. Before proceeding to MCA analysis, water quality parameters were statistically correlated with mortality rate to find the association between them. Key water quality parameters such as TAN, nitrite-N, and turbidity, and stocking density registered higher values than the optimal in the ponds affected with RMS, whereas other water quality parameters such as salinity, pH, hardness, alkalinity etc., did not have any correlation with healthy and RMS affected ponds. Based on the water quality data, these critical parameters and stocking density were categorized into low, medium and high in association with mortality variation, nil, up to 5% and above 25%, respectively (Table 7).

After having categorized the key water quality parameters, they were compared with the mortality rates. Out of 34 farms, 14 farms registered high turbidity (> 100 NTU) and showed > 25% mortality and four other farms with high turbidity showed < 25% mortality. Further, six farms which registered high turbidity did not suffer mortality (healthy ponds). Suspended solids in water could clog the gills, imposing stress to the animals and making them more susceptible to infections. TAN and nitrite N were found to be high in 13 and six farms which suffered > 25% mortality. In ponds with < 25% mortality, TAN and nitrite values were medium in 3 and 7 farms. While seven farms with medium stocking density (SD) showed < 25% mortality, six and four farms with high and medium SD showed > 25% mortality (Table 8).

Multiple correspondence analysis (MCA) was done with all the identified critical factors and mortality. The MCA plot (Fig.7) provides

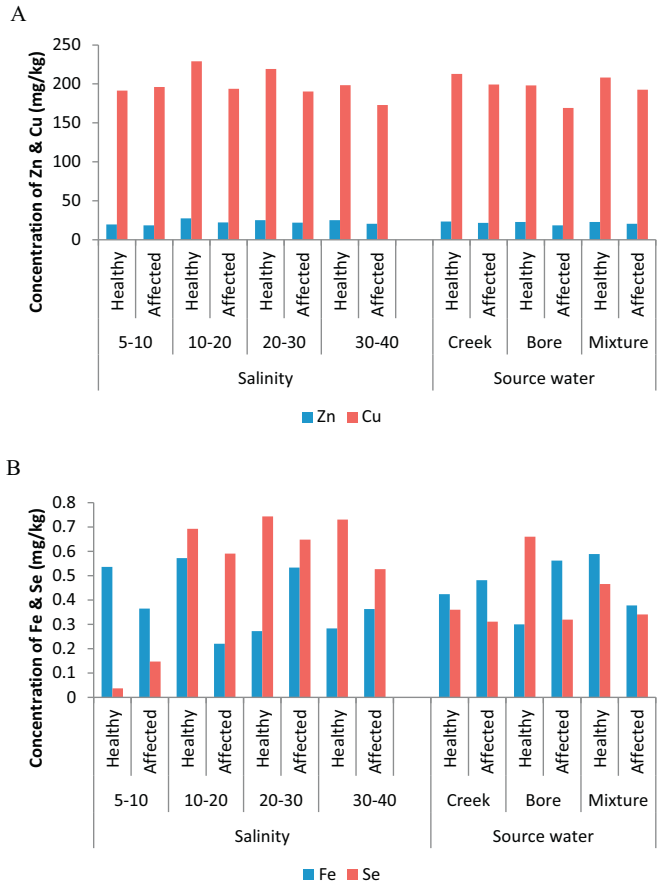

Fig. 6. Concentration of trace minerals in hemolymph of healthy and RMS affected shrimp cultured in varying salinities and different source waters: A. Zinc & Copper, and B. Iron & Selenium.

Table 7
Rating of critical parameters in relation to shrimp morality.

Critical parameter	Mortality rating (%)		
	Nil mortality	< 25% mortality	> 25% mortality
Tubidity (NTU)	< 25 (L)	25–100 (M)	> 100 (H)
TAN (ppm)	< 0.5 (L)	0.5–1.0 (M)	> 1.0 (H)
NO_2-N (ppm)	< 0.25 (L)	0.25–0.5 (M)	> 0.5 (H)
SD (Nos.m^{-2})	< 40 (L)	40–60 (M)	> 60 (H)

L: Low, M: Medium, H: High.

Table 8
Categorization of farms with respect to critical parameters and shrimp mortality.

Mortality rating (%)	Rating of critical parameter	No. of farms under respective critical parameter rating			
		Turbidity (NTU)	TAN (ppm)	NO_2-N (ppm)	SD (Nos.m^{-2})
0%	L	3	4	9	5
	M	0	5	0	4
	H	6	0	0	0
< 25% mortality	L	4	3	1	1
	M	1	3	7	7
	H	4	3	1	1
> 25% mortality	L	4	1	3	3
	M	0	2	7	7
	H	12	13	6	6

L: Low, M: Medium, H: High.

four quadrants and within the quadrants, the variables are associated and are different from other quadrants. Moderate level mortality was associated with medium stocking density, medium turbidity and medium nitrite-N (quadrant-I). No mortality was associated with low stocking density, medium turbidity and low nitrite N and low to medium TAN values (quadrant-II). High mortality was associated with high stocking density, high nitrite N, and high turbidity (quadrant-III). Quadrant-IV has no direct association with mortality and high TAN in this quadrant is very close to high mortality in 3rd quadrant (). The MCA plot clearly showed that it is not the single critical factor responsible for the mortality, but a combination of factors determining the mortality rates.

4. Discussion

The disease characterized by continuous low level mortalities (running mortality) in *P. vannamei* farms after about 35 days of culture has been causing significant concern among the shrimp farmers year after year, after its reported emergence in 2011 from shrimp farming hubs in Andhra Pradesh. Examination of affected shrimp for known and the OIE-listed pathogens has revealed that the disease is not caused by any these pathogens. The bacteriological findings indicated predominance of *Vibrio* species such as *V. parahaemolyticus* and *V. azurius* in the haemolymph of acutely affected shrimp from RMS affected ponds. *V. parahaemolyticus* isolates after confirmatory diagnosis using the VpM PCR and rRNA sequencing followed by AP-3 PCR indicates that these bacteria did not harbor the toxin genes present in the AHPND strains. Viral and bacterial metagenomic studies could be useful to identify unknown infectious agents involved if any. Since the bioassay and co-habitation experiments indicated that RMS was not an infectious disease, we did not carry out any metagenomic study. Haemolymphs collected from normal and affected shrimps were mostly sterile. Similarly, swab sample taken from the clinically visible affected muscle part did not show growth of bacteria and thereby ruling out the possibility of any bacterial disease due to *Vibrio harveyi* (Zhou et al., 2012; Soto-Rodriguez et al., 2012).

Further, the histological investigations on acutely RMS affected shrimp did not have significant pathologies. The pathology of hepatopancreas was grade-1 and was not characteristic of AHPND as explained by Tran et al. (2013). Melena et al. (2012) reported low mortalities associated with muscle necrosis in Ecuadorian *P. vannamei* shrimp farms and described macroscopic lesions involving opaque, whitish discolorations in the abdominal muscles, with lesions in skeletal muscle, including multifocal necrosis, fibrocytic inflammation and phagocytosis. Although, in the present study, muscle showed some necrosis associated with haemocytic infiltration, similar to IMNV affected shrimp presenting coagulative to liquefactive necrosis, accompanied by moderate infiltration and accumulation of hemocytes (OIE, 2014), failure to detect IMNV and PvNv by RT-PCR ruled out their involvement.

This RMS of shrimp under commercial production conditions continued through to harvest size. Usually, the mortality rate was slow (< 1%/day), but the cumulative loss over time was high, often reaching 70%, compelling the farmers to harvest the crops prematurely. We could not understand the progression from morbidity to death. As per some farmers, reducing feeding for a few days was found to help reduce shrimp mortalities. Some farmers reported adoption of best management practices involving reducing stocking density and feed management helped in reducing RMS problems.

It is very well proven that viral and bacterial diseases along with poor physico-chemical parameters are the prime cause for mortality in shrimp culture (Chamberlain, 1997). Earlier studies have proven significant influence of temperature on WSSV (Raj et al., 2012) and pH stress decreasing the resistance of white shrimp *P. vannamei* against *V. alginolyticus*, affecting the immune response (Li and Chen, 2008). Tedengren et al. (1988) explained that changes in salinity causes

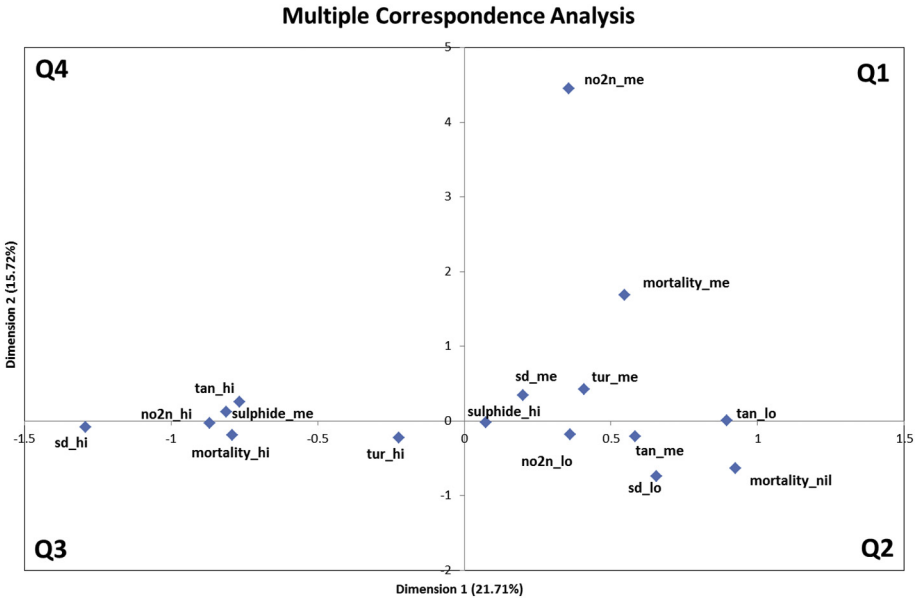

Fig. 7. MCA plot showing the shrimps mortality in association with critical parameters.

physiological stress which further decreases the tolerance to environmental stressors. Hence toxicants and environment stressors can act in synergistic manner possibly triggering pathogens from latency to virulence stage. It is well known that reduced conditions prove to be highly conducive for undesirable microbes such as sulfur bacteria through which reduced compounds like hydrogen sulfide is produced (Avnimelech and Ritvo, 2003). High level of organic carbon helps proliferation of heterotrophic bacteria as these bacteria consume the labile organic matter. Apart from the mineralization of organic matter, they consume considerable amount of oxygen thus influencing the water quality to a greater extent (Moriarty, 1997).

High sulfide values with maximum value of 0.174 ppm recorded in this study, is by far higher than the acceptable limits in aquaculture settings in RMS affected ponds. Generally, it is important to maintain pond bottom in oxidized state to get rid of reduced compounds, especially sulfide which should be always below 0.03 ppm in aquaculture ponds. The unionized form of sulfide can cross the lipid bilayer and block the respiration of the cultured animal (NRC, 1977). Apart from rendering direct toxicity, it also makes the cultured animal susceptible to bacterial and viral pathogens (Hsu and Chen, 2007).

In the present study, relatively higher levels of nitrite and TAN were recorded in ponds affected with RMS. TAN values in farms in West Godavari, Nellore and Nagapattinam districts of 1.305, 0.866 and 1.616 ppm respectively were much higher than the optimal levels. Further, nitrite N value of higher than 0.5 ppm in RMS affected ponds could have created stress to the animals. The effect of ammonia-N on physiological response or immune resistance of shrimp or other decapods have been well documented (Cheng and Chen, 2000; Liu and Chen, 2004). It is also very well proven that unionized ammonia can cause serious damage by crossing cell membrane and at even low values can become potentially toxic especially at higher pH. Liu and Chen (2004) studied the immune response of white leg shrimp and reported that high TAN levels made shrimp susceptible to *V. alginolyticus* infections.

Several studies have indicated that the stressors in aquaculture are mostly related to water and pond sediment quality (Lo and Kou, 1998). Takahashi et al. (1995) reported that water quality parameters such as pH, salinity, temperature and hardness make shrimp susceptible to pathogens. Significant difference in the soil and water quality parameters between disease affected and unaffected ponds have been also recorded (Krishnani et al., 1997). Stressors generally act on the immune system of cultured animals and may help multiplication of pathogens and the process of infection, causing associated mortalities. It is further documented that the mortality rate was low in low saline ponds and when the stocking density was reduced, farmers were able to harvest the crop without RMS. Over stocking generally deteriorates the water quality and increases the rate of disease transmission. Tendencia et al. (2010) studied WSSV outbreak in intensive farms along with water quality variables. Using logistic regression model, they concluded that pond environmental factors such as pH and temperature are considered as high risk factors for the infection but not necessarily for the disease outbreak. They also reported that the ponds with lesser water transparency are exposed to more stressful environment compared to ponds with higher water transparency. In the present study, as reported by farmers, temperature also found to be one of the important risk factor for the occurrence of RMS. The low feed intake by shrimps during high temperatures in ponds with high stocking density coupled with the critical water parameters beyond the optimal range resulted in the mortality of shrimps. We have also observed that combinations of critical environmental parameters are likely to make the animals more stressed and subsequently resulted in mortality compared to these parameters reported as singly by earlier researchers. MCA analysis results suggest that nitrite concentration above 0.5 ppm alone may not have resulted in mortality, but in combination with other critical parameters viz., TAN and turbidity might have resulted in high mortality per cent.

In the present study, the levels of most of the major and trace minerals were found to decrease in RMS affected shrimp. Variation in the concentrations of minerals in shrimp hemolymph has been observed under different stress conditions (Boglio, 1995). In case of shrimp, the ionic concentration of hemolymph is more or less similar as in the case of water. But the stress induced on the animals by exposing them to change in the water pH and TAN resulted in lowered mineral values. Chen and Chen (1996) reported that increased TAN concentration resulted in decreased hemolymph mineral concentration. Low level of magnesium can cause increase in respiratory rate, which could translate into reduced survival and poor growth rate (Roy et al., 2007). Potassium is an intracellular cation having an important role in electrolyte and acid-base balance of intracellular fluids (Gong et al., 2004; Cheng et al., 2006). The reduction of this cation also would have resulted in reduced survival in RMS affected ponds. Stress conditions showed lower sodium (Na^+) and Cl^- and higher K^+ concentration. It is reported that

injection with biogenic amines (dopamine) showed transient elevations in osmolality, Na^+ and Cl^- levels in *P.monodon* (Chang et al., 2007) and *P.vannamei* (Chiu et al., 2006; Liu et al., 2008). In the present study also, it has been observed that in RMS affected shrimp, the concentration of major and trace minerals decreased suggesting that the animals were stress conditions in most of the ponds. Recently it was reported that combined exposure of ammonia and nitrite to *Marsupenaeus japonicus* induced synergistic and simultaneous effects on respiratory parameters, the acid–base balance and osmoregulation (Chen and Cheng, 1996; Chen et al., 2013).

In shrimp aquaculture, high stocking densities coupled with use of large amount of feeds are being practiced to maximize productions. Many times due to poor pond management practices, critical water parameters might be beyond the optimal levels irrespective of stocking density. Organic matter that originates from unused feed and fecal material settle at the pond bottom, followed by of microbial metabolism, resulting in high amounts of nutrients and metabolites (ammonia, nitrate, nitrite, sulfide) in high stocking shrimp ponds (Joseph et al., 2001). Generally these are recycled either by native microorganisms or by application of microbial products. Exposure to stressors originating from pond water could compromise the immunity of aquatic organisms, thereby increasing the risk of infection and physico-chemical condition of pond may stimulate the proliferation of pathogens (Tendencia et al., 2012). Pond soil serves as reservoir for many harmful pathogens. Viral load in soil was reported to be of the orders of 10^9–10^{13} per gram which is several folds higher than overlying water (Breitbart and Rohwer, 2005). A number of studies have revealed the relationship between physico-chemical parameters and disease outbreak.

5. Conclusion

In the present study, investigations could not identify any known bacterial or viral pathogen responsible for the RMS. The cohabitation of RMS affected animals with healthy animals could not induce disease in healthy animals. Rearing of RMS affected animals in sea water with optimum environmental parameters resulted in complete recovery from the clinical symptoms of RMS.

Given the condition of RMS, it would have been best to collect continuous samples from the same pond throughout the prevailing system and get accurate information regarding correlation of water quality parameters with this syndrome. However, our main objective was to find out if any infectious agent is associated with this condition and therefore, we tried to collect as many samples possible from different locations with different ecosystems but representing different stages of this condition. Nevertheless, as sampling was done at different DOCs from several ponds, we tried to correlate the environmental parameters with RMS with this limited sampling conditions.

In the absence of any etiological agent associated with RMS in the present study, it appears that chronic shrimp mortality was due to interaction of several critical environmental parameters and stocking density higher than the optimum levels that are triggered under adverse environmental conditions. The results of this study makes us assume that the RMS is an aquaculture system associated syndrome, where mortality of shrimp occurs whenever critical factors in the system are not within the optimal ranges possibly due to poor management practices such as inadequate pond preparation and less time gap for the ponds to dry between the crops. However, a more systematic study is necessary to accurately predict this.

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