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Survival Kinetics of *Vibrio* Species in a Tropical Estuary along the Southwest coast of India - as a function of selected Environmental Factors

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Abstract

Vibrio species, autochthonous to aquatic environments worldwide, comprises of various species causing serious infections in humans and aquatic animals. Studying the survival ability of pathogenic *Vibrio* spp. in the aquatic environments is significant to identify the risk posed by them to aquatic animals as well as the recreational users of the system. However, in spite of their public health significance, the removal kinetics of this organism are not studied in detail from Cochin estuary, a highly productive estuarine system along the south-west coast of India. Here we aim to study the survival kinetics of pathogenic *Vibrio* spp. (*V. parahaemolyticus*, *V. mimicus*, *V. proteolyticus*, *V. alginolyticus* and *V. vulnificus)* in the estuary as a function of biological and physico-chemical factors based on microcosm experiments. All the five *Vibrio* spp. showed prolonged and better survival in the estuarine sediment compared to water. This indicates that the sediments of Cochin estuary may act as permanent repository of this bacterium and is a matter of serious concern. Biological factors and chemical composition of estuarine water revealed to play a significant role in the removal of *Vibrio*s from the estuarine environment. Exposure to sunlight also exhibited deleterious effect on the survival of these organisms. Thus, our overall findings reveal that the estuarine system has a self-purifying capacity to control the pathogens such as *Vibrio*. However, entry of high load of pathogens into the system through pollution and anthropogenic activities may disrupt this balance.

Keywords: Survival ability; *Vibrio*; Environmental factors; Sunlight; Sediment; Cochin estuary

Introduction

*Vibrio*s are Gram-negative halophilic bacteria that inhabits estuaries, marine environments and aquaculture settings worldwide (Thompson et al., 2004). At present the genus *Vibrio* comprises of more than 100 species (Okada et al., 2010) and many are recognized as human pathogens; associated with outbreaks of water and seafood-borne gastrointestinal infections in humans (Austin, 2010). The bacterium is also known to cause high morbidity, mortality, or infections in aquatic animals (Osunla & Okoh, 2017). Vibriosis caused by this bacterium is one of the most serious diseases in fishes and other aquatic animals and pose a major challenge to food security and economy of the country. Not all strains of *Vibrio* are pathogenic; they turn pathogenic under favourable environmental conditions. Survival of *Vibrio* in aquatic environments is dependent on various factors. The ecology of *Vibrio* species in aquatic environments have been found to be associated to a wide range of environmental factors mainly physical (sunlight, temperature), chemical (salinity, chemical composition of water) and biological (competition with autochthonous microflora, bacteriophage lysis and grazing by protozoan

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predators) and to the presence of organic matter, its habitat, and the location (Arunagiri et al., 2013). However, previous studies have shown that the effect of these environmental parameters on the bacterium is species dependent (Caburlotto et al., 2012).

The Cochin estuary is one of the largest and highly productive estuarine systems along the south-west coast of India (Vinita et al., 2013). It is rich in fishery resources and also a popular destination for recreational activities. The estuary is currently under the threat of nutrient enrichment by the anthropogenic interventions and terrestrial inputs through land runoff (John et al., 2020). The studies on survival kinetics of such *Vibrio* spp. from Cochin estuary and the role played by environmental factors in its natural elimination process is not attempted in detail till date. Such studies are very significant as this will give us a detailed insight about the extent of survival of the pathogen in estuarine environments. Prolonged persistence of such pathogens will have an adverse effect on the beneficial uses of the estuary. Here, we aim to evaluate the survival kinetics of *Vibrio* bacterium in the Cochin estuarine system based on various microcosm experiments.

Materials and Methods

Five *Vibrio* species previously isolated from Cochin estuary and maintained at room temperature in the fish pathology laboratory of Cochin University of Science and Technology were used for the study. This included *Vibrio parahaemolyticus* (Genbank Accession No. KM406325), *Vibrio alginolyticus* (KT005561), *Vibrio vulnificus* (KT005560), *Vibrio proteolyticus* (KT748656) and *Vibrio mimicus* (KT187246). The strains were previously screened for the presence of virulence factors and multi-drug resistance. All the five strains showed virulence potential as they harboured extracellular virulence enzymes and were multi-drug resistant. In addition, *V. parahaemolyticus* PM1S2 strain also harboured *T3SS1*, *tdh* and *trh* virulence genes (Silvester et al., 2017).

All the microcosms were designed using previously described protocol (Abhirosh et al., 2009). Water and sediments samples were freshly collected from Cochin estuary using Niskin sampler and Van veen grab respectively and brought immediately to the laboratory for setting up the microcosms. Water microcosms were prepared by adding 100 ml of the collected estuarine water into sterile 250 ml Erlenmeyer flasks. Sediment microcosms were prepared by adding 50 gm of collected sediment and 50 ml of overlaying estuarine water into sterile 250 ml Erlenmeyer flasks (Hood & Ness, 1982). This was done so as to mimic the estuarine condition.

Raw water (RW) and raw sediment (RS) were used to set microcosms to evaluate the effect of self-contained biotic factors present in estuarine water and sediment respectively on the survival of test organisms. Un-inoculated RW and RS microcosms were kept to find the presence of naturally occurring *Vibrio*, if any. Autoclave sterilised estuarine water (AW) and sediment (AS) devoid of all the self-contained biological factors such as protozoan predators, competing bacterial flora and bacteriophages were used to set control microcosms. All the microcosms were incubated in dark to nullify the effect of light.

About 500 mg l^{-1} of cycloheximide (eukaryotic inhibitor) was added into the raw water and sediment microcosm to study the individual role of protozoan grazing on the study organisms. The protozoans were identified by microscopic method.

Bacteriophages specific to *Vibrio* present in the water were detected by double layer agar method (Kennedy et al., 1986) using each of the 5 *Vibrio* species as host cells for bacterio-phage in separate experiments. The competing autochthonous bacterial flora in water and sediment was enumerated by the spread plate technique method on Nutrient agar (Himedia, India). The THB isolates were identified up to the genus level using various biochemical tests mentioned in the Bergey's manual of determinative bacteriology (Buchanan & Gibbons, 1984).

The effect of temperature and sunlight on the survival of test organisms were studied. Estuarine water (100 ml) freshly collected from the estuary and sterilised by autoclaving (AW) was used as test solution to evaluate the effect of temperature and sunlight. Three temperatures *i.e.,* 25°C, 30°C and 35°C were chosen for the study based on the range of temperatures observed in the estuary during the monsoon, post-monsoon and pre-monsoon seasons respectively. All the microcosms were incubated in dark. Glass bottles with test solution were inoculated with the test organisms and immersed in a 50 litres plastic tank (which act as a thermal jacket) containing estuarine water, and then kept exposed in the sun. The control microcosms were incubated

in dark to prevent exposure to sunlight. The experiment time was from 08 h till 18 h.

Filter sterilised (0.22 μm) estuarine water (to nullify the effect of biotic factors) was used as microcosm to study the effect of chemical composition of estuarine water on the survival of the test organisms. Isotonic saline was used as the control. To study the effect of salinity alone on the survival, autoclaved water with salinities adjusted to 0 ppt, 15 ppt and 30 ppt were used as test microcosms. Nutrients present in the estuarine water was analysed by Grassholf et al., 1983 and heavy metals were analysed by Brooks et al., 1967; Smith & Windom, 1972.

For preparation of inoculum, each of the test bacterial strain was aseptically inoculated into Tryptone Soy Broth and incubated for 24 h at 37°C. After incubation the bacterial cells were harvested by centrifugation at 3000 rpm for 5 min. The cell pellet was washed twice in sterile isotonic saline (0.85% NaCl) and resuspended in sterile isotonic saline to prepare the inoculum. One ml (approximately 10^8 CFU ml^{-1}) of each inoculum was added into the microcosms with 100 ml estuarine water or sediment plus water to give initial inoculum density of 10^6 CFU ml^{-1}.

Sampling and enumeration of the microcosms were done as follows. The bacterial count at the time of inoculation was taken as the initial count at time zero. Survival assay was carried out up-to 28 days with sampling at 1st, 3rd, 5th, 7th, 14th, 21st and 28th days. To study the effect of sunlight on test organisms, one-day experiment was set up with sampling at 2 h intervals. Samples were enumerated by the drop plate method (Hoben & Somasegaran, 1982). 10 μl of sample from each microcosm was drop plated onto a selective media for *Vibrio*; TCBS agar (Himedia, India) and also a non-selective media Tryptone Soy agar (Himedia, India). Two plating media in parallel such as the non-selective TSA and selective TCBS was used in order to understand the injury caused by the test solutions on *Vibrio* species, if any (Abhirosh et al., 2009). All the platings were done in triplicates, the plates were incubated overnight at 37°C and the number of colonies was counted and expressed as CFU/ml. The mean values from triplicate experiment were used to plot the survival curves using the Origin software.

Any significant difference in the survival of each test organism in different microcosms was analysed by T-test and one-way ANOVA in the SPSS statistical package. The significance level was set at 5%.

Results and Discussion

No growth was observed in the un-inoculated raw water and sediment microcosms, thus indicating the absence of naturally occurring culturable *Vibrio* in the estuarine water and sediment collected for microcosm preparation. A significant difference was observed in the survival of *V. parahaemolyticus*, *V. mimicus*, *V. proteolyticus*, *V. alginolyticus* and *V. vulnificus* in estuarine water and sediment ($p<0.05$) (Fig.1a-e). All the test organisms exhibited a prolonged and better survival in the estuarine sediment compared to the water. This may be due to high concentration of available nutrients in the sediment compared to water. Studies report that the marine sediments provide a natural protection to the bacteria against the bactericidal effect of the UV rays from the sun and also against predators (Abia et al., 2016). In a previous study, *V. fluvialis* survived better in the sediment microcosms when compared to water and this supports our findings (Amel et al., 2008). Thus, Cochin estuarine sediments may be permanent reservoirs for pathogens such as *Vibrio*. Similar reports are presented by (Abhirosh et al., 2011a, b) from a different study area.

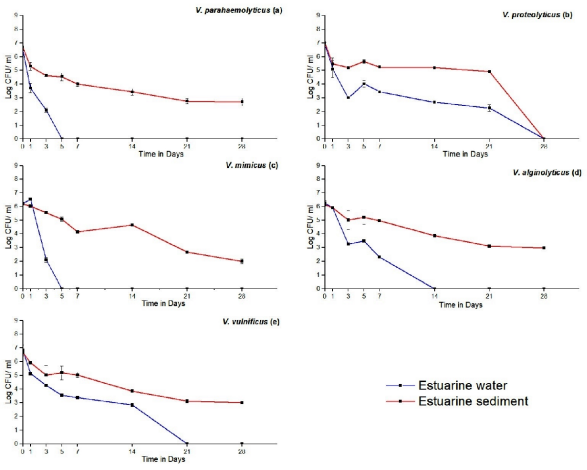

Fig. 1. Survival curves of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* in water and sediment of Cochin estuary

All the tested *Vibrio* species exhibited a better survival in the absence of biotic factors (AW, AS) compared to raw microcosms (RW, RS). A statistically significant difference ($p<0.05$) was observed in

the survival of all the five *Vibrio* species in raw and autoclaved water (Fig. 2 a-e) and sediment (Fig. 3 a-e). Though there was a difference in the survival pattern between each *Vibrio* spp., however, each of the species had a higher mortality rate in RW and RS microcosms with biotic factors (competing bacteria, bacteriophages and protozoans) compared to autoclaved microcosms AW and AS. The plate count of total heterotrophic bacteria (THB) isolated from water of Cochin estuary was 6 x 10^3 CFU ml^{-1} and a higher count was observed from sediment 2 x 10^8 CFU ml^{-1}. Bacterial genera identified from water were Gram-positive *Staphylococcus* and Gram-negative *Enterobacteriaceae, Flavobacterium* and *Moraxella.* Bacterial genera identified from the sediment were Gram-positive *Staphylococcus* and *Bacillus*, and Gram-negative *Alkaligenes, Cytophaga, Moraxella* and *Flavobacterium.* Bacteriophage specific to *V. parahaemolyticus* was isolated from the estuary and the mean phage count was 2×10^3 PFU ml^{-1}. Bacteriophages specific to the remaining four species were not detected in the estuary. The protozoans encountered in Cochin estuary were *Centropyxis, Difflugia, Euglena, Favella, Phacus, Tintinnids, Strombidium, Tintinnopsis, Stylonichia* and *Vorticella.*

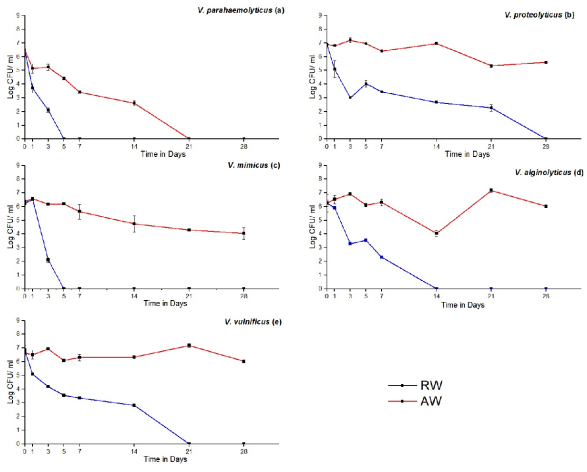

Fig. 2. Survival curves of (a) *V. parahaemolyticus*, (b) *V. proteolyticus*, (c) *V. mimicus*, (d) *V. alginolyticus*, (e) *V. vulnificus* as a function of biotic factors in water of Cochin estuary; RW: raw water, AW: autoclaved water

When the role of protozoan predation alone on the test organisms was studied, the effect was more visible in the microcosms treated with cycloheximide when compared to the untreated. The survival

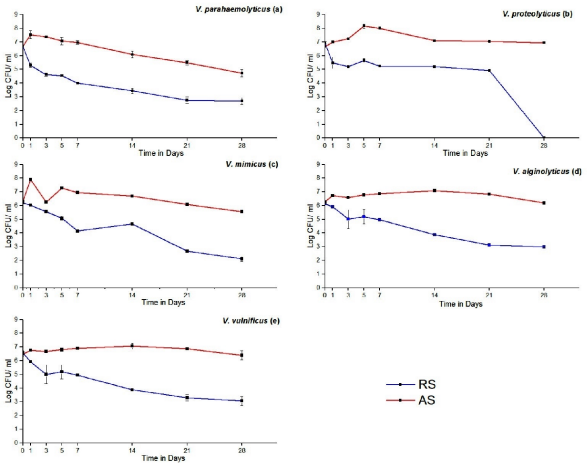

Fig. 3. Survival curves of (a) *V. parahaemolyticus*, (b) *V. proteolyticus*, (c) *V. mimicus*, (d) *V. alginolyticus*, (e) *V. vulnificus* as a function of biotic factors in sediment of Cochin estuary; RS: raw sediment, AS: autoclaved sediment

curves of five *Vibrio* species in cycloheximide treated water and untreated raw water (RW) microcosms are shown in Fig. 4 a-e. *V. parahaemolyticus* and *V. mimicus* showed a difference in the survival pattern in raw and treated microcosms. However, the difference was not statistically significant ($p>0.05$). *V. proteolyticus* showed a statistically significant difference in the survival pattern in treated and untreated water (RW) ($p<0.05$). Thus, a higher mortality was observed in the raw water microcosms that had protozoan predators. However,

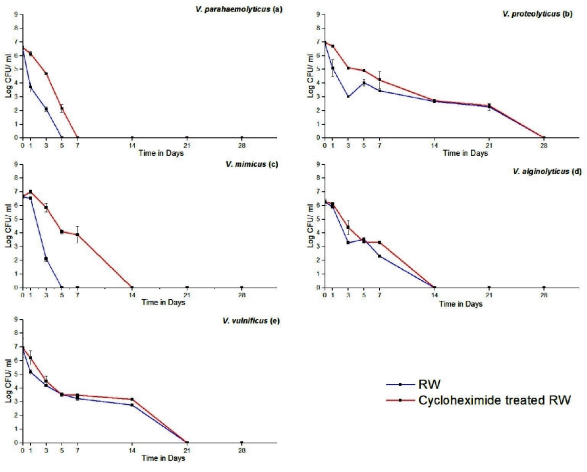

Fig. 4. Survival curves showing effect of protozoan predation on survival of (a) *V. parahaemolyticus*, (b) *V. proteolyticus*, (c) *V. mimicus*, (d) *V. alginolyticus*, (e) *V. vulnificus* in water of Cochin estuary; RW: raw water

protozoan predation did not show any significant effect on the survival of *V. vulnificus* and *V. alginolyticus* in water (p>0.05).

The survival curves of the five *Vibrio* spp. in cycloheximide treated and untreated raw (RS) sediment microcosms are shown in Fig. 5a-e. The difference in the survival pattern of the *V. proteolyticus, V. mimicus, V. vulnificus* and *V. alginolyticus* in the treated and untreated sediments were not statistically significant (p>0.05). More or less similar pattern was observed in the treated and untreated microcosms. *V. parahaemolyticus* showed a statistically significant difference in the survival in treated and control sediment (p<0.05). The direct effect of cycloheximide on the test organisms was negligible. Results clearly highlight the deleterious role played by various biotic elements in removal of *Vibrio* from the estuarine environment. Thus, our findings point out the significant part played by protozoans, phages and competing flora in controlling the survival of *Vibrio* in estuarine systems. Our results are in agreement with a study on removal *of V. parahaemolyticus* from Vembanad Lake (Abhirosh et al., 2009).

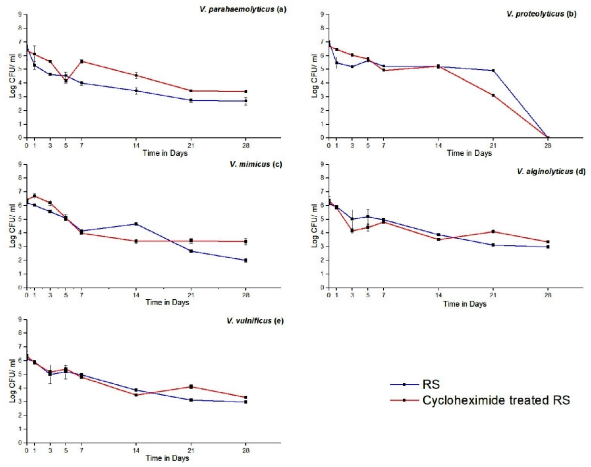

Fig. 5. Survival curves showing effect of protozoan predation on survival of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* in sediment of Cochin estuary; RS: raw sediment

The survival curves of each species at varying temperatures are given in Fig.6 a-e. All the test organisms showed a better and extended survival at lower temperatures compared to elevated temperature. However, the variations in the survival of the five species at 25°C, 30°C and 35°C was not statistically significant (p>0.05). In our study, we could find that lower temperatures favoured extended survival of vibrios. This is in agreement with previous studies highlighting extended survival of bacteria in fresh and marine waters at lower temperatures (An et al., 2002). Studies also emphasize that the effect of various environmental conditions will vary from one species of *Vibrio* to another (Osunla & Okoh, 2017).

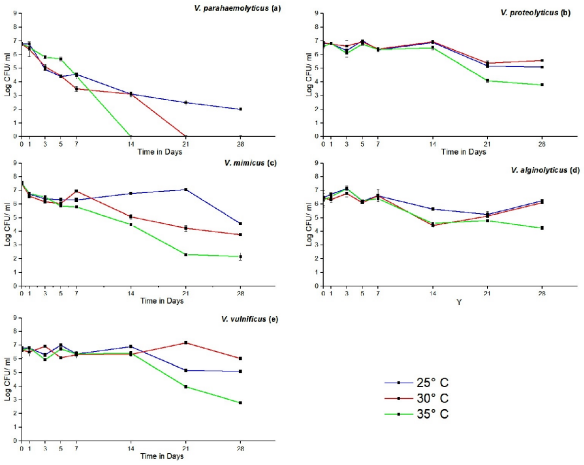

Fig. 6. Survival curves showing effect of different temperatures on survival of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* in Cochin estuary

When the effect of sunlight was tested, *V. parahaemolyticus* was more tolerant to sunlight compared to the other four species tested. There was no significant difference in the survival of *V. parahaemolyticus* in the presence and absence of sunlight (p=0.6) and more or less similar survival pattern was observed (Fig. 7 a-e). Sunlight had a statistically significant deleterious effect on the survival of *V. mimicus*, *V. proteolyticus*, *V. vulnificus* and *V. alginolyticus* (p<0.05). Their survival was better in microcosms kept in dark compared to those exposed to sunlight. The sunlight intensity measured was maximum at 14 h (98 kilolux) and the least was observed at 18 h (4 kilolux). Previous studies states that sunlight plays a major role in the inactivation of pathogenic bacteria in aquatic environments (Abhirosh & Hatha, 2005; Anuar & Chan, 2013).

The chemical composition of the estuarine water also exhibited a significant deleterious effect on the survival of all the tested *Vibrio* spp. The differences in the survival of all five species in the test and

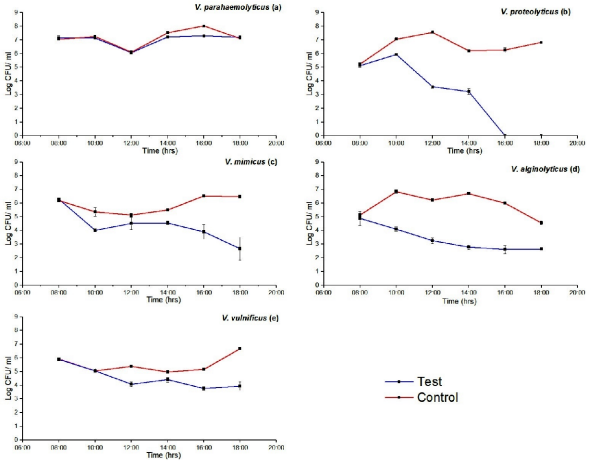

Fig. 7. Survival curves showing effect of sunlight on survival of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* in Cochin estuary

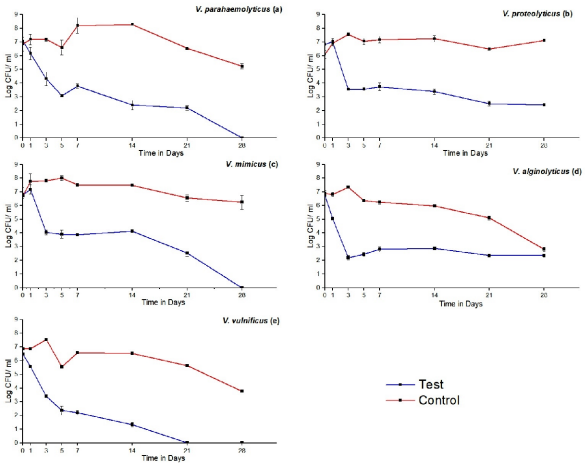

Fig. 8. Survival curves of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* as a function of chemical factors in water of Cochin estuary

control microcosms were highly significant (pd=0.002). All the five species exhibited a relatively extended survival in the control microcosms devoid of chemical factors compared to the test microcosm until the end of the experiment (Fig. 8a-e). The nutrient levels in the estuarine water were ammonia-5.73 mg L^{-1}, phosphate-5.16 mg L^{-1}, nitrite-0.34 mg L^{-1}, nitrate- 1.50 mg L^{-1} and silicate-10.37 mg L^{-1}. The chemical factors such as secondary metabolites (antibiotics secreted by autochthonous bacteria), heavy metals, toxins, etc. might be responsible for this bactericidal activity in the aquatic systems (Sharma, 2000). The concentration of heavy metal in the estuarine water were Pb-0.0045 ppm, Cd-0.00045 ppm, Fe-0. 93724 ppm, Ni-0.00635 ppm, Zn-0.01135 ppm. Sb, Cr, Hg and As were below detection limit. A previous report also pointed out this estuary to be polluted with such heavy metals (Bindu et al., 2015).

A high prevalence of *Vibrio* is usually observed during summer months (high saline condition) (Oliver, 2010). In our study, all five species showed an enhanced survival at higher salinities (Fig. 9 a-e). However, the difference in survival curves of *V. parahaemolyticus*, *V. alginolyticus, V. proteolyticus,* and *V. mimicus* at 0 ppt, 15 ppt and 30 ppt were not significant statistically ($p>0.05$). *V. vulnificus* showed an enhanced survival at 30 ppt followed by 15 ppt and the least at 0 ppt. There was a significant difference in the survival of this species at different salinities ($p<0.05$). While analysing the effect of varying salinities on the survival of the species, we could thus find a better survival at higher salinities (15ppt and 30 ppt) when compared to zero saline condition. *Vibrio* spp. except *V. cholerae* and *V. mimicus* are halophilic in nature. At lower salinities the membrane integrity is lost and the bacterium remains under stress (Deepanjali et al., 2005).

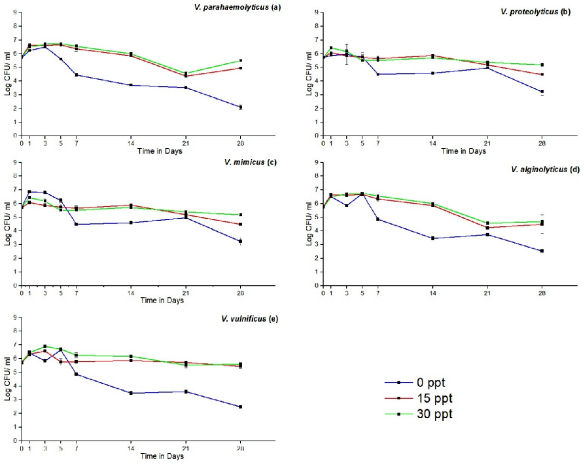

Fig. 9. Survival curves of (a) *V. parahaemolyticus,* (b) *V. proteolyticus,* (c) *V. mimicus,* (d) *V. alginolyticus,* (e) *V. vulnificus* at varying salinities

The extended persistence of pathogenic species of *Vibrio* in Cochin estuary is a serious concern for health of recreational and commercial users of the system and also for the aquatic animals. Even though the study highlights the role played by biological, physical-and chemical factors in the self-

purification of Cochin estuary, entry of high load of pathogens into the system through anthropogenic activities may disrupt this natural balance. Hence, the local health authorities must initiate necessary measures to control pollution, so as to prevent any potential future outbreaks in the area and also to guarantee safety of the fish and shellfishes.

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