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## **Daily variations in pathogenic bacterial populations in a monsoon influenced tropical environment**

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### **Abstract:**

Changing climatic conditions have influenced the monsoon pattern in recent years. Variations in bacterial population in one such tropical environment were observed everyday over two years and point out intra and inter annual changes driven by the intensity of rainfall. *Vibrio* spp. were abundant during the monsoon and so were faecal coliforms. *Vibrio alginolyticus* were negatively influenced by nitrate, whereas, silicate and rainfall positively influenced *Vibrio parahaemolyticus* numbers. It is also known that pathogenic bacteria are associated with the plankton. Changes in the abundance of plankton, which are governed mainly by environmental changes, could be responsible for variation in pathogenic bacterial abundance during monsoon, other than the land runoff due to precipitation and influx of fresh water.

**Key words:** Estuary, Monsoon, Pathogenic bacteria, *Vibrio* spp., *Enterococcus faecalis*, Total coliforms

## 1. INTRODUCTION

Estuaries are anthropogenic hotspots and are also exposed to natural environmental perturbations. Estuaries influenced by seasonal variations in fresh water flux provide a window for change in the functioning of these ecosystems. The west coast of India experiences the south west monsoon. During the monsoon season estuaries receive increased fresh water discharge and the characteristics of the water body change. Bacterial populations, an important component of the microbial loop, influence food web dynamics and ecosystem functioning. The response of the bacterial population to the changing environment is rapid and an assessment of the health of such an ecosystem benefits from high resolution observations. Virulent pathogenic *Vibrio* species are expected more frequently in tropical marine environments, since the virulence gene expression seems to increase at elevated environmental temperatures (Mahony et al., 2010). Hence tropical coastal waters with year round temperatures near 30° C would ensure most favourable growth conditions for these pathogens (Reichardt et al., 2013). Coliform pathogens from faecal contamination have received public and scientific attentions for more than a century and have been regarded as one of the most important indicators for monitoring pathogenic bacteria (Li et al., 2014).

In this study we measured variations in the abundance of *Vibrio* species (*Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*), faecal pathogens (*Enterococcus faecalis*, *Escherichia coli* O157:H7) and Total coliforms at Dona Paula bay, a semi enclosed bay, located at the mouth of the Zuari estuary (15°27'N, 73°48'E) along the central west coast of India. Zuari estuary receives an average rainfall of ~ 2500 mm of which 80% occurs during June–August. The study area experiences three seasons (1) South west monsoon (June-September) when the area is inundated with riverine and land runoff due to heavy precipitation and experiences stratification due to high influx of fresh water. (2) A post monsoon season (October-January) which is a recovery period from fresh water domination with an increase in salinity but little change in surface water temperature and (3) a pre monsoon season (February-May) when the water becomes typically marine (Manoj and Unnikrishnan, 2009) . A drop in the surface salinity is observed from 35.1 psu in premonsoon to 12 psu during southwest monsoon (Unpublished data).

## 2. MATERIALS AND METHOD

### 2.1 Sampling

Water samples were collected everyday (between 10:00 and 11:00 h) from July 2009 to August 2011 from Dona Paula bay which is located at the mouth of the Zuari estuary situated on the central west coast of India (Fig. 1). Subsequently, the samples were diluted as required, spread plated (0.1ml) on Zobell Marine Agar 2216 (Hi-media) and incubated at  $(30 \pm 2 \text{ }^\circ\text{C})$ , which was the water temperature of this estuary, for enumerating culturable bacteria. Zobell Marine Agar 2216 allows the growth of culturable, halotolerant and halophilic bacteria.

### 2.2 Quantification of bacteria

The pathogenic bacteria were quantified using specific media (Hi-media) following the manufacturer's instructions (Hi-media). The culture media were prepared using aged seawater from the study area. The diluted samples were spread plated on Thiosulphate-Citrate-Bile Salts (TCBS) for *Vibrio* spp. (sucrose fermenting, yellow, entire, raised <2 mm dia colonies were counted as *V. cholerae*, yellow coloured colonies, >2mm were counted as *V. alginolyticus* and green colour colonies as *V. parahaemolyticus*). Mac Conkey Agar was used for Total Coliforms (Included *E. coli*, *Salmonella* and *Shigella*). Gram negative bacteria usually grow well on the medium and are differentiated by their ability to ferment lactose. Lactose fermenting *E. coli* grows as red or pink and may be surrounded by a zone of acid precipitated bile. Lactose non-fermenting strains, such as *Shigella* and *Salmonella* are colourless and transparent. Enterococcus Confirmatory Agar was used for *E. faecalis* which grows as blue colour colonies, *Pseudomonas Aeromonas* selective agar for *Aeromonas/Pseudomonas* and HiCrome EC0157:H7 selective Agar for *E. coli* O157:H7 (ECO157:H7) which grows as purple colour colonies.

All the plates of specific media were incubated at 37°C for 24 h and colonies were counted. In order to reduce the uncertainties associated with counting of the pathogenic bacteria, at least 150 pure isolates of each of the pathogenic bacteria were randomly picked from the selective agar and were confirmed using a series of appropriate biochemical tests.

The bacterial species were also confirmed using MALDI-TOF MS Biotyping (MTB), which can be used alone or in combination with other proteomic tools, and has recently become established as a common technique for the detection, identification, and characterisation of micro-organisms (Emami et al. 2012).

This method can be complementary to the acquisition of data obtained from 16S rRNA gene sequencing with the added benefit of generating unique biochemical fingerprints for the sub-typing of species.

The culturable bacterial abundance (Total Viable Count, TVC) is expressed as CFU ml<sup>-1</sup>. The samples to be analysed for total bacterial count (TBC) (including culturable and non-culturable bacteria) were fixed with formaldehyde (final concentration 1 to 2%; v/v). The quantification of bacteria was performed using acridine orange and epifluorescence microscopy (Daley and Hobbie 1975) and the values are expressed as cells ml<sup>-1</sup>.

### 2.3 Nutrient analysis

Identifying the role of nutrients in the bacterial distribution, water samples were also collected for the nutrient analysis from 1<sup>st</sup> June 2010 to 31<sup>st</sup> August 2011. The concentration of different dissolved inorganic nutrients such as nitrate (NO<sub>3</sub>-N), nitrite (NO<sub>2</sub>-N), phosphate (PO<sub>4</sub>-P) and silicate (SiO<sub>4</sub>-Si) were determined using an autoanalyser (SKALAR ANALYTICAL SAN PLUS 8505 INTERFACE v 3.31). The rainfall (RF, mm) data were obtained from the Indian Meteorological Department, Panaji, Goa.

### 2.4 Data analysis

The relationship between the environmental variables (nutrients, rainfall) and bacteria was investigated by means of Canonical Correspondence Analysis (CCA), using the CANOCO version 4.5 (ter Braak and Smilauer, 2002). Bacterial abundance data were log (x+1) transformed. A forward selection was performed on the set of environmental variables that explained most of the variance and statistical significance of each variable was tested using a Monte Carlo permutation test under the reduced model with 999 numbers of permutations. The bacterial species showing relation with the environmental variables in the CCA analysis were also subjected to linear regression analysis to determine whether the correlation between specific bacterium and measured environmental variables were statistically significant.

## 3. RESULTS AND DISCUSSION

The total viable count (TVC) and total bacterial count (TBC) point out intra annual variations (Fig. 2a,b). The viable bacterial abundance (TVC) ranged from  $1.5 \times 10^1$  to  $2.9 \times 10^6$  CFU ml<sup>-1</sup> (Fig 2.a), whereas the total bacterial count varied from  $1.9 \times 10^5$  cells ml<sup>-1</sup> (July 2009) to  $5.9 \times 10^7$  cells ml<sup>-1</sup> (February 2011) (Fig. 2b). Inter annual variations point out that the total bacterial abundance increased

from 2009 to 2011, while the viable bacterial numbers decreased. Complex physical, chemical, and biological processes govern estuarine environments. Many of these parameters vary over diel light cycles, daily tidal cycles, rainfall events and seasonal change (Moss et al. 2006). Increase in total bacterial numbers observed in this study can be related to variations in rainfall and can be attributed to inputs from fresh water runoff from land. In this study it was found that nitrate increased considerably in the pre monsoon (Table 1). The study site is a bay that receives terrestrial organic inputs from the estuarine environments fringed with thick mangroves. The west coast of India experiences upwelling during the summer monsoon, leading to enhancement of nitrate (Naqvi et al., 2006). In this study we observed that at the mouth of the estuary nitrate concentration surged during the pre monsoon season and this was coupled with the increase in the phosphate and bacterial numbers (Table 1). The phosphate levels increased further in the monsoon 2011 with additional increase in the bacterial numbers. It has been reported earlier that the net heterotrophy leads to release of dissolved inorganic phosphorous to the ocean and dissolved inorganic carbon to the atmosphere (Smith and Hollibaugh, 1993; Sarma et al., 2001).

Seasonal observations in the study area during 2002-2003 reported higher pathogens during the monsoon and total coliforms ranged from 1 to 587 CFU/ ml (Rodrigues et al., 2011). An earlier investigation in the study area pointed out the influence of sewage on the pathogenic bacterial abundance (Nagvenkar and Ramaiah, 2009). In this study, we observed that they were present throughout the study period, maximum in August 2010 and varied from 0 -  $7.6 \times 10^5$  CFU ml<sup>-1</sup> (Fig. 3a). There was a wide variation in the concentration of total coliforms and TVC both showing a decreasing trend from year 2010 to 2011. The percentage contribution of total coliforms with respect to total viable bacterial abundance (TC:TVC) reduced from 26% to 0.2%. Waterborne outbreaks of pathogenic bacteria from contaminated water are serious threats for public health and coliform bacteria have been regarded as one of the most important indicators for monitoring pathogenic bacteria. To address potential pathogenic bacterial outbreaks, the distribution and diversity of coliform bacteria in Jiahe river, which flows through a densely-populated urban area in China, was assessed. The results indicated that temperature, oxidation-reduction potential (ORP) and Total bacterial abundance (TBA) were three main parameters impacting the distribution and diversity of coliform bacteria, which is important for predicting and preventing waterborne transmission of pathogenic bacteria (Li et al., 2014).

In this study, *Aeromonas/ Pseudomonas* were detected only in the month of August 2009 and abundances ranged from 0 – 10 CFU ml<sup>-1</sup>. No studies have been conducted in this area regarding the *E.*

*coli* O157:H7 strain which is a pathogenic strain. The occurrence of *E. coli* O157:H7 was the highest ( $2.55 \times 10^3$  CFU ml<sup>-1</sup>) in the month of September 2009, however, no influence of rainfall or other environmental parameter was observed on their abundance (Fig. 3b). The faecal contaminants, *E. faecalis* were seen only during September 2009 and July 2010 respectively (Fig. 3c). Due to a sharp rise in the human settlement in the coastal zones, the surrounding aquatic environment, which is used for various activities such as fishing and recreation, are subjected to a rise in the distribution of human pathogenic microbial forms. The tendency of these bacteria entering water is greater during monsoon owing to huge fresh water runoff. Members of *Aeromonas* spp., *Escherichia coli*, and *Enterococcus* spp., along with many others have also been reported from zooplankton samples (Dixon, 2005). A recent study reported that zooplankton contain high numbers of pathogenic bacteria such as *V. cholerae*, *E. coli* and *S. faecalis* (Khandeparker and Anil, 2013).

*V. cholerae* (VC) ranged from 0 –  $2.28 \times 10^5$  CFU ml<sup>-1</sup>. In August 2011, highest numbers of VC were observed (Fig. 4a). *V. alginolyticus* (VA) ranged from 0 –  $5 \times 10^4$  CFU ml<sup>-1</sup> (Fig. 4b). *V. parahaemolyticus* (VP) ranged from 0 –  $1 \times 10^5$  CFU ml<sup>-1</sup>. Highest abundance was observed in August 2011 (Fig. 4c). The average silicate level during monsoon 2010 was highest and rainfall was lower when compared to monsoon 2011. The study site is a shallow tropical bay, physical processes during monsoon (freshwater discharge) and non-monsoon seasons (currents, waves and tides) cause re-suspension of benthic diatom propagules, the blooming of which depends upon nutrient availability (P, Si, NO<sub>2</sub> & NO<sub>3</sub>). Variations in monsoon (D'Costa et al., 2008) and break period influence the phytoplankton dynamics (Patil and Anil, 2011) in this region. The increase in phytoplankton production in turn would influence the higher trophic level i.e. zooplankton. A recent study by Khandeparker et al (2014) in the same region also indicated that the monsoon period is characterised by higher diatom and bacterial abundance in the biofilms compared to other seasons. Many studies have reported association between *V. cholerae* and chitinous plankton in the marine environment (Huq et al., 1983; Gil et al. 2004) and found that *V. cholerae* and *V. parahaemolyticus* preferentially attach to planktonic copepods. Some of them are important pathogens for fish (*V. alginolyticus*) and humans (*V. cholerae* and *V. parahaemolyticus*) and are closely linked with diatoms (Rehnstam-Holm et al., 2010). Although all the *Vibrio* spp. were present year-round, probably due to their close association with the plankton, they were more abundant during monsoon.

CCA was used to relate the distribution of different pathogenic bacteria to environmental variables. The analysis pointed out that the total bacterial abundance (TBC) was influenced by phosphate (Fig. 5a,b) in

the monsoon of 2011 ( $p = 0.07$ ). Silicate and rainfall positively influenced the total viable *V. parahaemolyticus* (VP) numbers (Fig. 5a,b) mainly in the monsoon of 2010; significant correlation was also observed between silicate and VP ( $p = 0.03$ ) and rainfall and VP ( $p = 0.04$ ). *V. alginolyticus* was negatively influenced by nitrate ( $p=0.04$ ) during the post monsoon 2010 and pre-monsoon 2011 (Fig. 5a). Nutrient concentrations have been shown to be relevant for *Vibrio* spp. (Rehnstam-Holm et al., 2010 and references therein). Phytoplankton and bacteria both play key roles in the carbon, nitrogen and phosphorus cycles (Lin et al., 2006). Recently Hitchcock and Mitrovick (2014) demonstrated that discharge plays an important structuring role for carbon, nutrient and bacteria dynamics on the Bega and Clyde river estuaries, in Australia. In this study P, Si,  $\text{NO}_2$  &  $\text{NO}_3$  were chosen as bacteria play a major role in N and P cycles and are influenced by them, whereas silicate can be used as a proxy for diatoms, a major component of the microphytoplankton whose population can influence both bacterial composition and abundance (Khandeparker et al., 2014). A study carried out in the Chesapeake Bay reported that hindcast prediction yields an improved understanding of environmental conditions associated with occurrence of *V. vulnificus*, an estuarine bacterium, which is a causative agent of seafood related gastroenteritis, primary septicemia and wound infections worldwide (Banakar et al., 2011). All the pathogenic bacteria monitored in the present study are known to be closely associated with the plankton (phytoplankton and zooplankton). Variations in the abundance of plankton are governed mainly by environmental changes. These could be responsible for changes in pathogenic bacterial abundance during monsoon other than the land runoff due to precipitation and influx of fresh water. Vibrios are ubiquitous in the marine environment and under normal circumstances mediate important ecological functions; however changes in environmental factors such as salinity, temperature, pH and nutrients can trigger expression of the virulence genes (Singleton et al., 1982; Lara et al., 2009). Studies addressing virulence gene expression in relation to changes in environmental characteristics should be taken up to move this field forward.

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Legends to Table:

Table 1: Average values of rainfall, silicate ( $\text{SiO}_4\text{-Si}$ ), phosphate ( $\text{PO}_4\text{-P}$ ), nitrite ( $\text{NO}_2\text{-N}$ ), nitrate ( $\text{NO}_3\text{-N}$ ), and bacterial population (Total Viable Count (TVC), Total Bacterial Count (TBC), *E. coli* O157:H7 (EC), Total Coliforms (TC), *E. faecalis* (EF), *V. cholerae* (VC), *V. alginolyticus* (VA) and *V. parahaemolyticus* (VP)).

Legends to Figures:

**Fig. 1.** Map showing Dona Paula bay located at the mouth of the Zuari estuary situated on the central west coast of India.

**Fig. 2.** Daily variation in (a) Total Viable Count - TVC (b) Total Bacterial Count – TBC and (c) Rainfall from 6<sup>th</sup> July' 2009 to 31<sup>st</sup> August' 2011. Blocked area represents monsoon period.

**Fig. 3.** Daily variation in (a) Total Coliforms - TC, (b) *E. coli* O157:H7 - EC (c) *E. faecalis* - EF and (d) Rainfall from 6<sup>th</sup> July' 2009 to 31<sup>st</sup> August' 2011. Blocked area represents monsoon period.

**Fig. 4.** Daily variation in (a) *V. cholerae* - VC (b) *V. alginolyticus* - VA (c) *V. parahaemolyticus* – VP and (d) rainfall from 6<sup>th</sup> July' 2009 to 31<sup>st</sup> August' 2011. Blocked area represents monsoon period.

**Fig. 5.** Canonical correspondence analysis (CCA) for bacterial abundance in the monsoon, post monsoon and pre monsoon in surface waters together with environmental variables in Dona Paula Bay, West Coast of India (a) Days biplot with the environmental variables (b) Bacterial species biplot with environmental variables.

Only the five environmental variables that explained most of the variance are included in the analysis (i.e. Rainfall, nitrate ( $\text{NO}_3\text{-N}$ ), nitrite ( $\text{NO}_2\text{-N}$ ), phosphate ( $\text{PO}_4\text{-P}$ ) and silicate ( $\text{SiO}_4\text{-Si}$ )).

**Table 1**

	Monsoon 2010	Post Monsoon 2010	Premonsoon 2011	Monsoon 2011
Rainfall (mm)	11.5 ± 18	2.1 ± 6	0.04 ± 0.2	18 ± 17
Silicate (µmole L <sup>-1</sup> )	35 ± 16	21 ± 11	9.5 ± 6	28 ± 9.6
Phosphate (µmole L <sup>-1</sup> )	2.5 ± 2	1.7 ± 1.3	6.4 ± 9.8	9.9 ± 7.5
Nitrite (µmole L <sup>-1</sup> )	1.5 ± 1.6	3 ± 2	11 ± 9	0.4 ± 0.2
Nitrate (µmole L <sup>-1</sup> )	4 ± 3.9	3.5 ± 2.3	23.3 ± 31.5	3.9 ± 3.1
Total Viable Count (CFU ml <sup>-1</sup> )	100909 ± 53000	56500 ± 48713	72496 ± 96515	49218 ± 50918
Total Bacterial Count (Cells ml <sup>-1</sup> )	1962769 ± 542570	2718700 ± 4049488	14517684 ± 7466987	12533145 ± 5567912
<i>E. coli O157:H7</i> (CFU ml <sup>-1</sup> )	3 ± 18	0	1 ± 2	0
Total Coliforms (CFU ml <sup>-1</sup> )	26549 ± 121456	3126 ± 27246	65 ± 217	119 ± 341
<i>S. faecalis</i> (CFU ml <sup>-1</sup> )	73 ± 96	0	0	0
<i>V. cholerae</i> (CFU ml <sup>-1</sup> )	6 ± 36	6 ± 62	3 ± 17	0
<i>V. alginolyticus</i> (CFU ml <sup>-1</sup> )	2 ± 9	16 ± 121	14 ± 91	0
<i>V. parahaemolyticus</i> (CFU ml <sup>-1</sup> )	112 ± 1009	14 ± 101	6 ± 25	46 ± 150

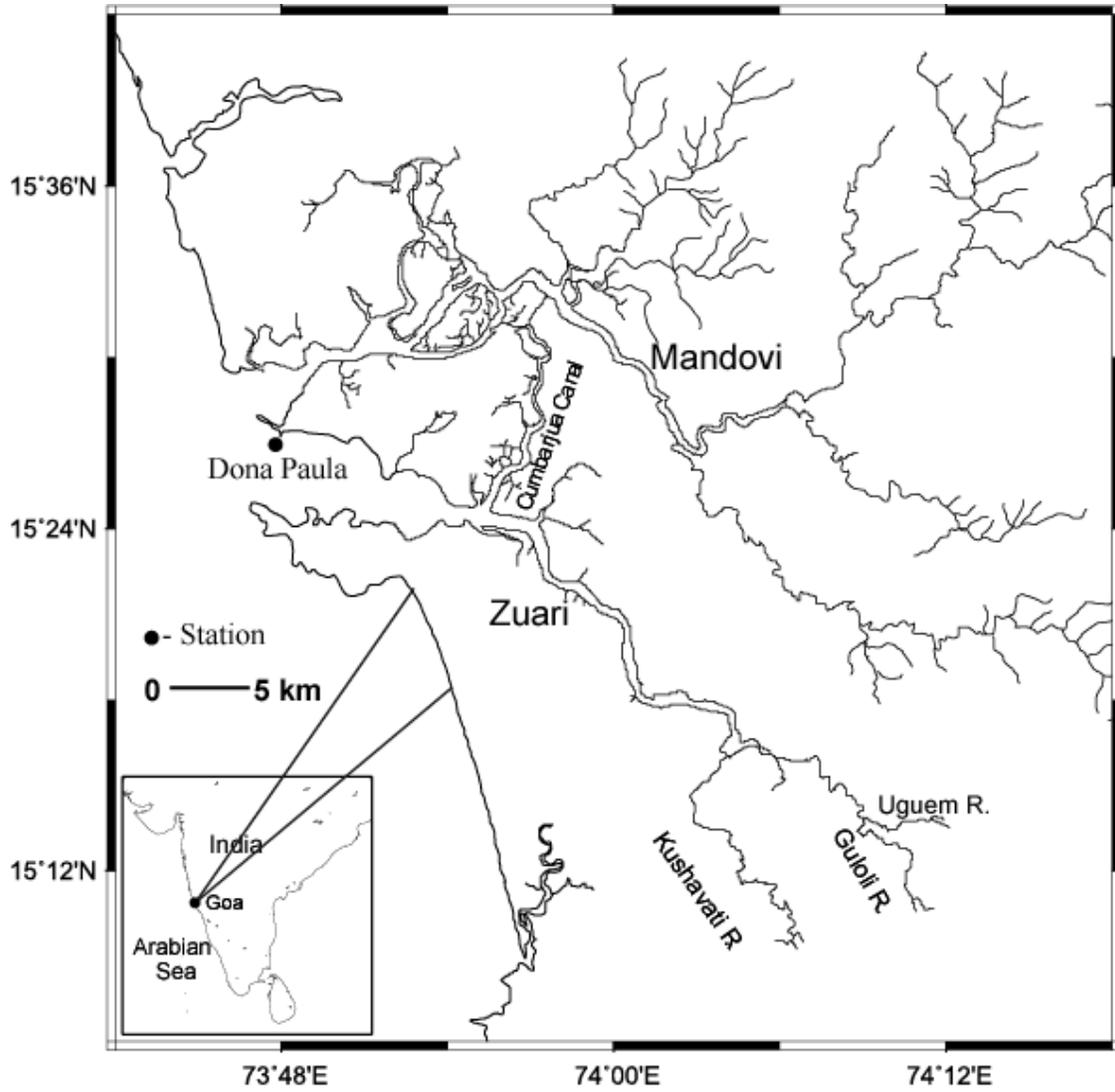


Figure 1

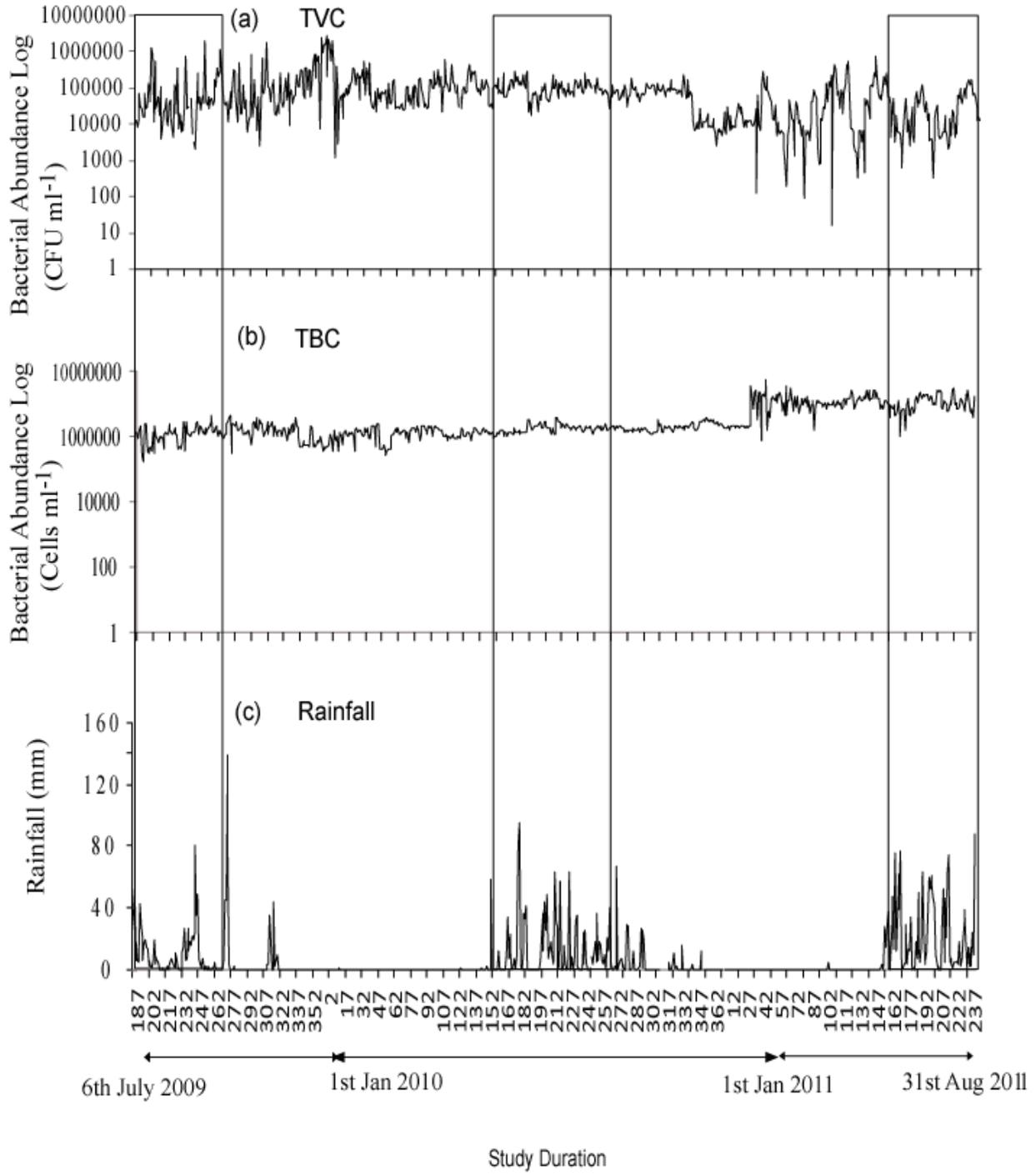


Figure 2

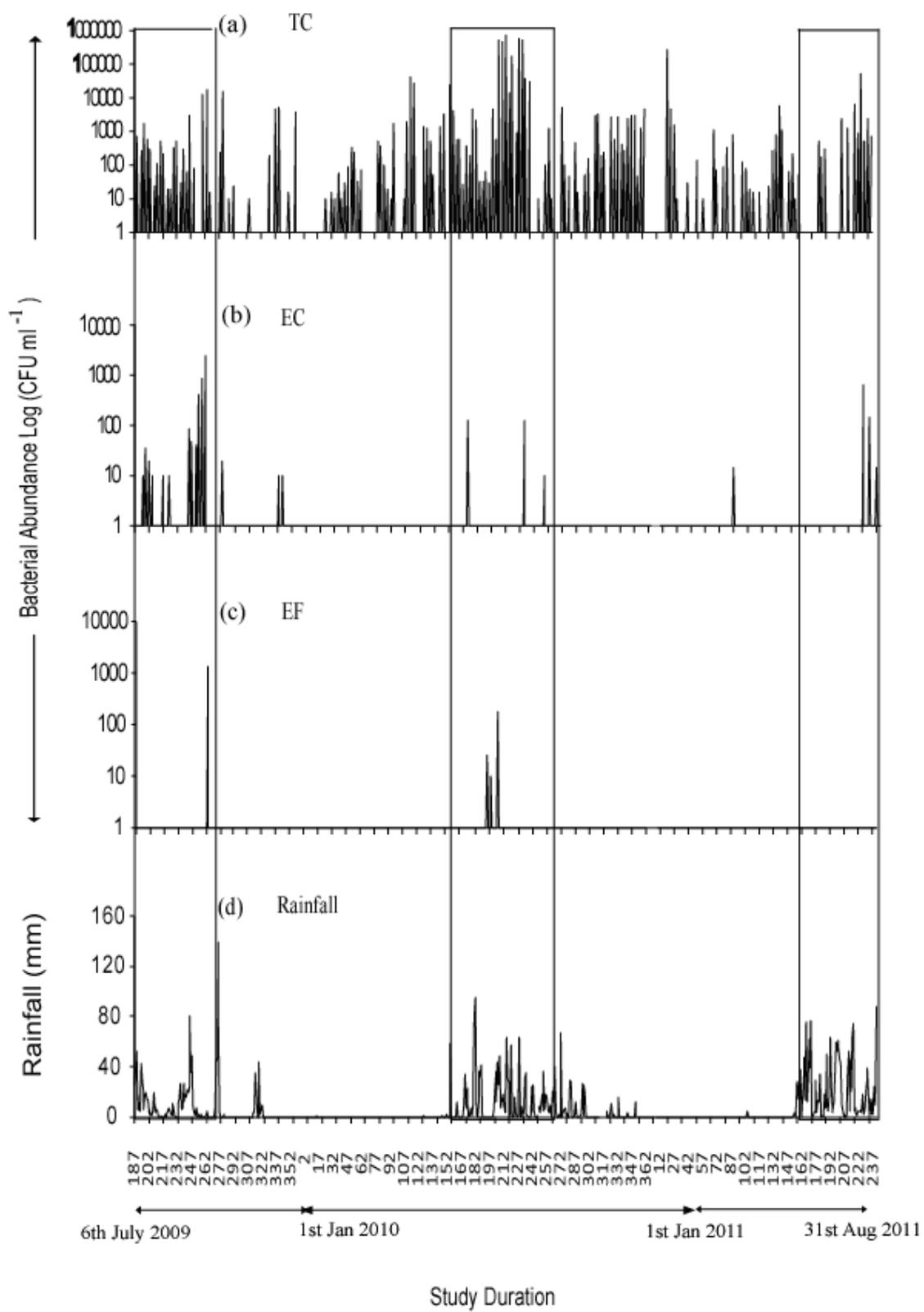


Figure 3

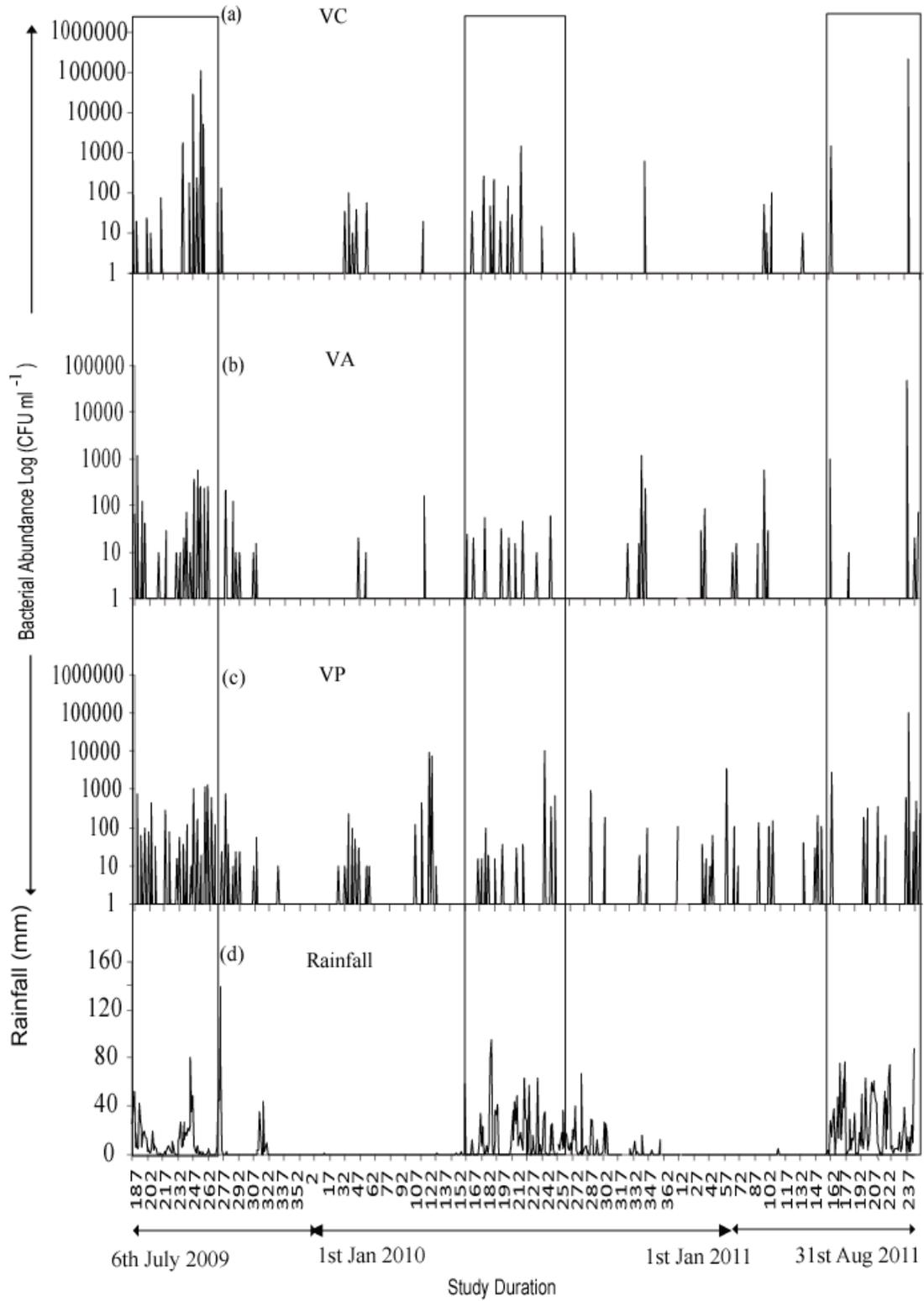


Figure 4

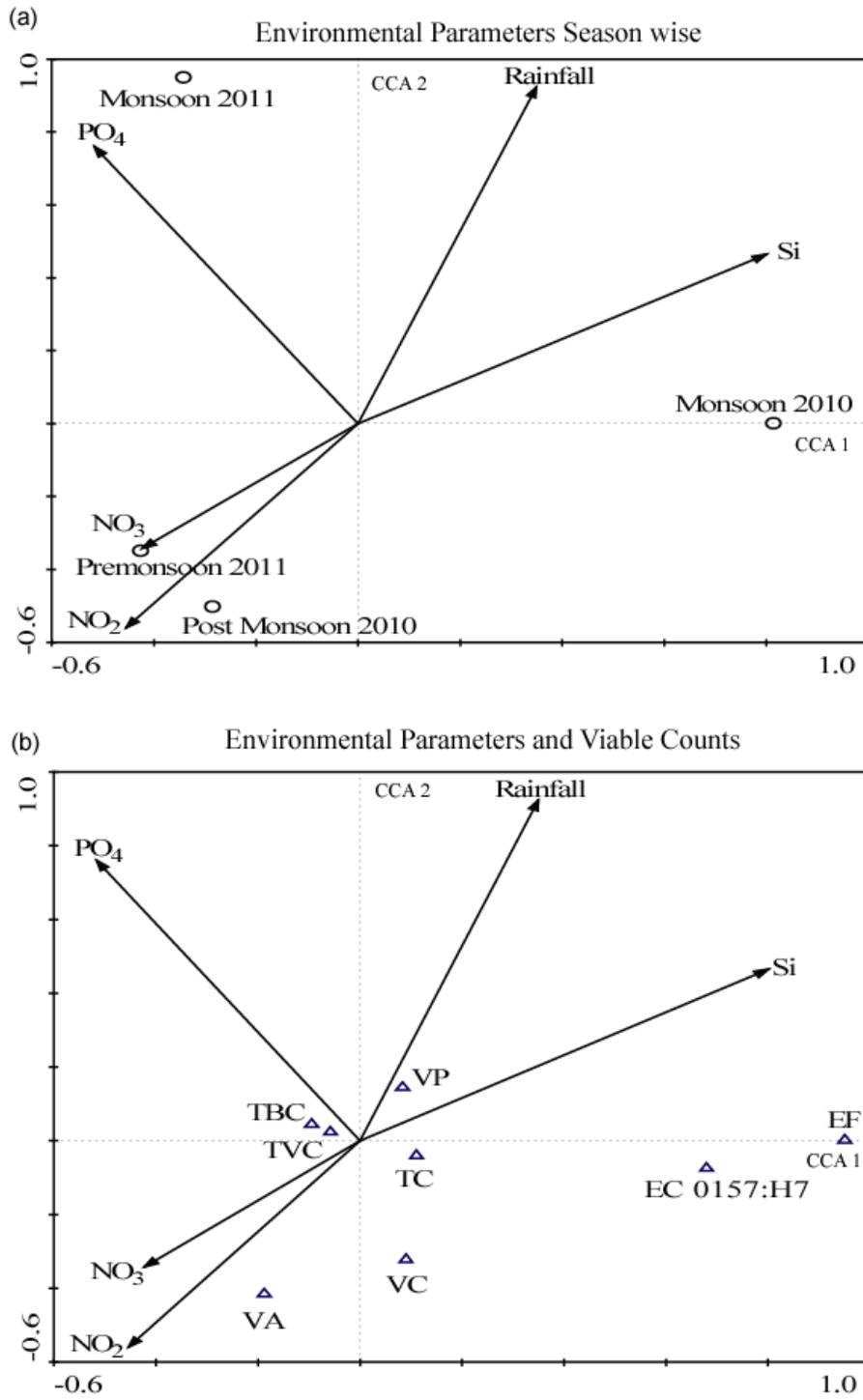


Figure 5