

**Inter-annual variability of phytoplankton assemblage and *Tetraspora gelatinosa* bloom from anthropogenically affected harbour, Veraval, India**

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**Abstract**

Veraval, one of Asia's largest fishing harbours, situated on the south-west coast of Gujarat, India has transformed into an industrial hub dominated by fish processing units, rayon manufacturing industry and transportation facilities. The study investigated the high abundance of *Tetraspora gelatinosa* along with the augmented level of ammonia in the harbour. The high concentration of ammonia was associated with the accumulation of sewage, industrial and fishery wastes in the harbour. Low energy expenditure associated with assimilation of ammonia made it a principal nitrogen source for *Tetraspora gelatinosa* growth. Even though ammonia is the preferred nitrogen source by phytoplankton, elevated concentration causes toxicity to the cells. Augmented level of ammonia and high TSS hampered the efficiency of PS II thereby impeding the chlorophyll *a* degradation and oxygen evolution. Built of the organic load from fish processing industries as well as domestic waste along with a reduction in photosynthetic oxygen evolution has made the harbour hypoxic ( $DO < 1.6 \text{ mg L}^{-1}$ )/anoxic ( $DO = 0.0 \text{ mg L}^{-1}$ ). Shannon-wiener diversity index as a pollution index suggested that the inner harbour area was highly polluted as the diversity ranged from 0.01 to 1.57. Whereas the outer harbour (Near-shore and off-shore) with less anthropogenic effect recorded high diversity (av. 2.17) suggesting a healthy environment.

**Keywords**– Phytoplankton, *Tetraspora gelatinosa* bloom, Hypoxia, Harbour, India

## **Introduction**

In recent decades, rapid growth in industrialization and urbanization in coastal zones have imposed a host of anthropogenic stresses on nearshore ecology which has exerted a detrimental influence on several aquatic ecosystems. To support the needs of ever-growing population land-based anthropogenic inputs has been increasing around the globe (Seitzinger et al. 2010). Consequently, most of the fresh water, as well as coastal ecosystem throughout the world, are facing the serious problems of eutrophication (Smith et al. 2006). Increased nutrient inputs, largely through domestic wastewater and runoff from fertilized agricultural fields, cause a predictable increase in the abundance of noxious and toxic harmful algal bloom (HAB), changes in the phytoplankton species composition and even hypoxia in wetland and marine ecosystem (Smith 2003; Seitzinger et al. 2010).

Dissolved oxygen depletion (hypoxia) is a widespread phenomenon and is reportedly growing globally (Gillbert et al. 2009; Ram et al. 2014). Earlier reported studies are Gulf of Mexico (Scavia et al. 2003), northern Adriatic Sea (Giani et al. 2012), Chesapeake Bay (Hagy et al. 2004), Neuse River Estuary (Eby and Crowder 2002), Tapi Estuary (Ram et al. 2014) and Arabian sea (Gomes et al. 2014). The dissolved oxygen concentration at which various fauna suffocate varies, but adverse effects on biota are perceived at a concentration below 2 mg L<sup>-1</sup> (Diaz 2001). Hypoxia may also affect predator-prey interaction and food web structure (Kemp et al. 2009).

The Gujarat state on the northwest coast of India has the longest coastline (1600 km) among the Country's maritime states with a wide variety of aquatic flora and fauna. It is one of the major fish producing maritime state and topped the list with 723,000 t marine fish production in 2016-17 (Sundararajan et al. 2017). Veraval harbour which is located on the south-west side of the Gujarat coast significantly contributes to the State's landings. It is a port town with one of the largest fishing harbours in Asia (Sundararajan et al. 2017). Several fish processing industries that export high-quality seafood to United States, Southeast Asia, Japan, Europe and the Gulf are located in the vicinity of the Veraval harbour making the export of seafood one of the major sources of income to the population. One of India's largest rayon manufacturing industry and several medium and small-scale industries are also located on the coast of Veraval. The town's domestic wastewater and effluents from some of these industries are released in the harbour basin deteriorating its water quality. The anthropogenic organic load entering the harbour seems to be in excess of its assimilative capacity and the water of Veraval remains hypoxic/anoxic irrespective of tide and season. Reduction

of sulphate has given rise of  $\text{NH}_4^+\text{-N}$  and  $\text{H}_2\text{S}$  concentration in the harbour water (Majithiya et al. 2017), the presences of which is felt even in the surrounding air.

Green algae are considered an excellent source of biofuel as a renewable energy source. Maneeruttanarungroj et al. (2010) found that *Tetraspora gelatinosa* can produce hydrogen gas and can be used as a model strain for photobiological hydrogen production. Apart from this, Gazala et al. (2004) reported the presences of saturated/unsaturated fatty acids, antibacterial and antifungal activity in two species of *Tetraspora* sp.

Earlier, *Tetraspora* sp. was reported from polar, sub-polar, temperate, subtropical, equatorial and tropical region (Richter et al. 2014 and references therein). In India, it was reported from Maharashtra (Lohar and Korekar 2015), Cochin (Dayala et al. 2014), Andhra Pradesh (Kaprapu and Rao 2013), Jharkhand (Toppo and Suseela 2013), Uttarakhand (Malik and Bharti 2012) and Karnataka (Shanthala and Shankar 2009). In this regard studies were mainly focused on the occurrences but the physico-chemical properties of *Tetraspora gelatinosa* habitat was studied sparsely (Vinocur and Pizarro 2000; Ritcher et al. 2014). However, the bloom report of *Tetraspora gelatinosa* has not yet cited in any of the literatures.

Worldwide there are several reports on phytoplankton from fishing as well as shipping harbour ports viz., Manazuru harbour, Japan (Satoh et al. 2000), Egg harbour, New Jersey (Olsen and Mahoney 2001) Eastern harbour, Egypt (Labib 2002; Ismael 2003), Los Angelis harbour, California (Schnitzer et al. 2007). From India, the studies have been conducted from states like Odisha (SubhaRao 1969), Maharashtra (Ramaiah et al. 1998), Kerala (Jugnu 2006), Karnataka (Harnstrom et al. 2009), Andhra Pradesh (Baliarsingh et al. 2012) and Goa (Patil and Anil 2015). The information regarding various biotic and abiotic parameters from Veraval harbour is limited. Studies conducted by Bhadja et al. (2014) on the effect of industrial development on seawater quality; Borade et al. (2015) on the correlation between physico-chemical parameters and bacterial indicators; and by Mandal et al. (2015) on the bloom of Coccolithophore (*Discosphaera tubifera*) are the only available information from this area. Studies related to phytoplankton community dynamics with respect to environmental drivers are deficient from the Veraval harbour.

This study was aimed to address: 1) the occurrence of *Tetraspora gelatinosa* bloom in the marine environment of Veraval harbour, 2) the spatio-temporal variation of phytoplankton diversity and community composition 3) status of physico-chemical parameters during the study period and its implication on phytoplankton assemblage. Since several fish processing and other industries are located in the vicinity of Veraval harbour, it is hypothesized that the *Tetraspora gelatinosa* bloom in

the Veraval harbour is governed by the allochthonous nutrient inputs. This is the first bloom report of *Tetraspora gelatinosa* from the marine environment, therefore its morphological and molecular confirmation is also provided.

## **Material and Method**

### **Site description**

Veraval is located at 20.9° N and 70.37° E on the south-west coast of Gujarat, India (Fig. 1). Fish processing and its export are the major activities performed at the Veraval coast which constitute a significant source of economy to the population.

Sampling was carried out at seven locations off Veraval, constituting of the inner harbour (V1 and V2- which receives high anthropogenic inputs), near-shore (V3, V4 and V5- along the shore) and off-shore (V6 and V7- coastal stations). The seasonal samples were collected in the month of March (Pre-monsoon), September (Monsoon) and December- January (Post-monsoon) from the study region during the period 2011 to 2015. The 2013 year represents post-monsoon sampling (single time).

### **Analytical methods**

The samples for the analysis of physico-chemical and biological parameters were collected through power boat equipped with eco-sounder (Garmin GNX 20) using a 5-L Niskin water sampler (Hydro-Bios, Kiel-Holtenau, Germany). Handheld thermometer (Brand, U.K.) was used for measurement of temperature. Salinity, dissolved oxygen (DO; mg L<sup>-1</sup>) and Total suspended solids (TSS; mg L<sup>-1</sup>) were estimated by titration and a gravimetric method respectively (Grasshoff 1983; APHA 2005). Dissolved inorganic nutrients like nitrite (NO<sub>2</sub><sup>-</sup>-N; μmol L<sup>-1</sup>), nitrate (NO<sub>3</sub><sup>-</sup>-N; μmol L<sup>-1</sup>), ammonia (NH<sub>4</sub><sup>+</sup>-N; μmol L<sup>-1</sup>) and phosphate (PO<sub>4</sub><sup>3-</sup>-P; μmol L<sup>-1</sup>) were analysed spectrophotometrically according to the protocol of Grasshoff (1983). The phytoplankton biomass (Chlorophyll *a*; mg m<sup>-3</sup>) was analysed fluorometrically (Turner Trilogy Model 7200, USA) before and after acidification following the standard protocol (Strickland and Parsons 1972). Lugol's fixed phytoplankton samples were identified morphologically under a compound light microscope (Motic BA 410, 400 magnifications) using taxonomic key illustrated in Subrahmanyam 1946; Desikachary 1959; Tomas 1997 and Richter et al. 2014. The enumeration of phytoplankton was carried out using Sedgwick rafter chamber (PYSER- SGI Ltd. UK) and their density was calculated as per the UNESCO 1994 protocol. The detailed information of methods used for analysis of biotic and abiotic parameters have been described elsewhere (Hardikar et al. 2017).

## Statistical analysis

For systematic and statistical interpretation of results, the biotic and abiotic datasets were processed with the aid of appropriate statistical software. Different univariate (Shannon-Wiener diversity index ( $H' \log_2$ ), Pielou evenness ( $J'$ )) and multivariate analysis (Spearman's rank correlation matrix ( $r$ ), principal component analysis (PCA) and multidimensional scaling (MDS)) were applied to find out the phytoplankton diversity and environmental drivers of *Tetraspora gelatinosa* as well as phytoplankton assemblages. Spearman's rank correlations were computed using XLSTAT 2016, statistical software to assess the correlation between the environmental variables and phytoplankton communities. Using XLSTAT 2016, PCA analysis was carried out to examine the environmental variables influencing the major phytoplankton. MDS was performed using Bray-Curtis similarity index to investigate the potential grouping of samples with respect to time (Period of sampling) or location (stations) based on square root transformed species abundance data (Field et al. 1982). The analysis was performed using the PRIMER v 6.1.4 software package (PRIMER-E Ltd.). Phytoplankton diversity index ( $H'$ ) and evenness ( $J'$ ) were calculated using primer v 6.1.4 (Clarke and Warwick 1994).

## Identification of *Tetraspora gelatinosa* using scanning electron microscopy (SEM)

*Tetraspora gelatinosa* (*T. gelatinosa*) sample of 5 ml was taken into 10 ml capacity plastic tube and cells were fixed by adding 2 ml of 0.5% osmium tetroxide (dissolved in filtered seawater) and incubated at 4°C for 5 minutes (Bistricki and Munawar 1978). After incubation, cells were filtered through 10 ml syringe connected to a small opaque plastic container (Swinnex) holding a 25 mm diameter, 0.45 µm nucleopore polycarbonate filter paper. The filtration was followed by 2-3 times washing with distilled water to remove excess salts. After washing the cells were dehydrated by passing through a series of increasing concentration of ethanol (25%, 50%, 75%, 95% and 100%). The last dehydration step with 100% ethanol was repeated twice. After the dehydration procedure, the filter paper was transferred to 1:2 solutions of hexamethyldisilazane (HMDS) and 100% ethanol followed by 2:1 solution of the same combination for 10 minutes. In the end, the filter paper was transferred to 100% HMDS for 20 minutes for two times. After this, the filter paper was kept overnight in the covered or loosely capped condition in a fume hood to allow HMDS to evaporate. For SEM analysis the filter paper was mounted on an aluminium stub and coated with gold (2 nm) using Polaris sputter coater (Polaron SC620). The cells were observed using JOEL IT 300 (Singapore) SEM.

## **Plankton DNA extraction, PCR amplification and sequencing of 28S r RNA gene**

Plankton genomic DNA was isolated from strain RKHB (strain name assign for *T. gelatinosa*) using Genelute plant genomic DNA kit (Sigma- Aldrich). Extraction of DNA was confirmed by gel electrophoresis. The 28S rRNA gene fragments were amplified by PCR. The primers tetraF and tetraR were designed using primer design software. They were as follows:

tetraF: TCGAGACTAAGCCTTTGGCG

tetraR: CCCATGTCCAATTGCTGTTCA

The amplification was carried out in 25 µl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), MgCl<sub>2</sub> (25 mM), the primers tetraF (10 ppm) and tetraR (10 ppm), Taq polymerase (Sigma) (2U) and autoclaved Milli-Q water (Millipore) for 35 cycles of 94 °C for 60 s, 55 °C for 45 s and 72°C for 60 s, 72 °C for 7 minutes with initial 05 min denaturation at 94 °C using a PCR-Express thermal cycler (Applied Biosystems). The DNA sequences of the PCR products were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer) using the protocol recommended by the supplier. Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems). Databases (Gen Bank) were searched for sequences similarity analysis of the 28S rRNA sequence obtained. The sequences obtained were aligned with clustral x and the tree was built by using MEGA 4.

## **Result**

### **Physico-chemical parameters**

The spatio-temporal variation in physico-chemical parameters from 2011-2015 is presented in Fig. 2. Temperature did not show significant spatio-temporal variation. Comparatively lower salinity was recorded in the inner harbour stations (V1 and V2) than the near-shore and off-shore stations. The pH ranged between 7.1 and 7.8 at the inner harbour with comparatively lower values than near-shore and off-shore stations. TSS, on the contrary, revealed an opposite trend with pH, displayed high values at the inner harbour (av. 121.9 mg L<sup>-1</sup>) and low at the near-shore (av. 56.4 mg L<sup>-1</sup>) and the off-shore (av. 37.5 mg L<sup>-1</sup>) stations. DO displayed a remarkable spatial variation. The inner harbour stations V1 & V2 sustained anoxic (DO = 0.0 mg L<sup>-1</sup>) and hypoxic (DO < 1.6 mg L<sup>-1</sup>) condition which decreased towards near-shore (av. 5.3 mg L<sup>-1</sup>) and off-shore (av. 6.3 mg L<sup>-1</sup>) stations. While nutrients, namely NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NH<sub>4</sub><sup>+</sup>-N and PO<sub>4</sub><sup>3-</sup>-P exhibited remarkable spatial variation. NO<sub>2</sub><sup>-</sup>

-N and  $\text{NO}_3^-$ -N exhibited an increasing trend from the inner harbour to the off-shore stations, whereas  $\text{NH}_4^+$ -N concentration showed a decreasing trend. Inner harbour stations showed continual high  $\text{NH}_4^+$ -N concentration which ranged from 17.6 to 1233.7  $\mu\text{mol L}^{-1}$  (av. 412.9  $\mu\text{mol L}^{-1}$ ) than near-shore (av. 43.5  $\mu\text{mol L}^{-1}$ ) and off-shore (av. 5.5  $\mu\text{mol L}^{-1}$ ) stations.  $\text{PO}_4^{3-}$ -P followed the similar trend as that of the  $\text{NH}_4^+$ -N.

### **Phytoplankton assemblage and bloom report of *T. gelatinosa***

The enhanced growth of *T. gelatinosa* in the Veraval harbour explained the role of nutrients in their uncontrolled proliferation. Phytoplankton assemblage in the harbour was composed of chlorophytes, diatoms, dinoflagellates and cyanophytes. The inner harbour stations were always dominated by chlorophytes accounting for almost 80% of the total population. Among chlorophytes, *T. gelatinosa* was the dominant genera contributing 90% to the total chlorophytes density (Fig. 3). While the near-shore and off-shore stations were dominated by diatoms throughout the study period.

Phytoplankton biomass in terms of chlorophyll *a* (Chl. *a*) in Veraval harbour ranged from 1.0 to 15.9  $\text{mg m}^{-3}$  (av. 5.2  $\text{mg m}^{-3}$ ) and Phaeophytin (Phae.), the degraded form of Chl. *a* which ranged between 0.6 and 30.2  $\text{mg m}^{-3}$  (av. 5.6  $\text{mg m}^{-3}$ ). The inner harbour stations recorded low Chl. *a* compared to phae. and the ratio (Chl. *a* : Phae.) was  $<1$  except during 2014. Whereas, the near-shore and off-shore stations, exhibited the opposite scenario with Chl. *a* : Phae. ratio  $>1$  (Fig. 4).

The longterm spatial trend of *T. gelatinosa* density from 2011 to 2015 revealed that the stations located inside the harbour sustained high density which decreased towards near to off-shore stations (Fig. 5). Since 2011, the temporal analysis revealed that the cell density at stations V1, V2 and V3 was  $7.91 \times 10^6$  cells  $\text{L}^{-1}$ ,  $6.88 \times 10^6$  cells  $\text{L}^{-1}$  and  $1.46 \times 10^6$  cells  $\text{L}^{-1}$  respectively which increased to  $45.93 \times 10^6$  cells  $\text{L}^{-1}$ ,  $26.86 \times 10$  cells  $\text{L}^{-1}$  and  $19.46 \times 10^6$  cells  $\text{L}^{-1}$  at the respective stations by 2015. A four fold increase in *T. gelatinosa* density at each locations was established during the study period. While towards the off-shore region the *T. gelatinosa* density decreased gradually showing its complete absence at off-shore stations. The high abundance of *T. gelatinosa* imparted reddish-brown colour to the water in the Veraval harbour.

### **Morphological and genetic Identification**

Based on scanning electron microscope (SEM) study, the detailed morphological features of *T. gelatinosa* were analysed. The study revealed that the cells occurred in a group of two or four surrounded by a transparent sheath. The cells were spherical to oval in shape with a cell size of 1–2  $\mu\text{m}$  (Fig. 6). The habitat ecology for the optimal growth of *T. gelatinosa* is low mesotrophic, cold

water in cool temperate, subpolar and polar zone where they can grow up to 9  $\mu\text{m}$  to 10  $\mu\text{m}$  (Richter et al. 2014). The considerable high temperature in the tropical zone in comparison to the temperate, polar and subpolar region would explain the smaller cell size of the *T. gelatinosa* in the Veraval harbour (Richter et al. 2014). Since there was a disparity regarding the habitat ecology of *T. gelatinosa* the amplified 28S rRNA sequence was compared to the 28S rRNA sequence in the gene bank. The results indicated the identity of 98% with *Tetraspora* UTEX-LB 234 (Gen Bank accession no. AF395514). Therefore, the strain RKHB was considered as one of the strains of *T. gelatinosa* (Gen Bank accession no. KY055012). The phylogenetic tree based on the 28S rRNA gene of strain RKHB depicts the position of strain within the genus *T. gelatinosa* (Fig. 7).

### Univariate and multivariate analysis

Shannon- Wiener diversity index  $H'$  ( $\log_2$ ) varied considerably in Veraval harbour and ranged from 0.01(V1) to 3.95 (V4), whereas Pielou's evenness index  $J'$  varied between 0.00 (V1) and 0.78 (V4). Both these indices are directly proportional to each other and followed a similar trend. The lowest  $H'$  occurred at the inner harbour stations where *T. gelatinosa* was in high abundant. Interestingly, with decreasing *T. gelatinosa* density, the phytoplankton diversity ( $H'$ ) increased from near to off-shore stations (Fig. 8).

The significant Spearman's rank correlation ( $p \leq 0.05$ ) between environmental variables and *T. gelatinosa* density is presented in Table 1. Significant positive correlations of *T. gelatinosa* density with  $\text{NH}_4^+\text{-N}$  ( $r = 0.82$ ,  $p < 0.0001$ ) and  $\text{PO}_4^{3-}$  ( $r = 0.77$ ,  $p < 0.0001$ ) suggested that the nutrient enrichment favoured the proliferation of this species in the Veraval harbour which in turn contributed significantly to the TSS ( $r = 0.53$ ,  $p < 0.001$ ). Further, the negative correlation obtained between *T. gelatinosa* density and  $\text{NO}_3^-\text{-N}$ , DO, salinity and pH revealed that these parameters were inversely related to or got hampered by *T. gelatinosa* bloom.

PCA was performed on major phytoplankton genera and environmental parameters as depicted in Fig. 9. PCA analysis showed that  $\text{PO}_4^{3-}\text{-P}$ , DO, pH, salinity,  $\text{NO}_3^-\text{-N}$ , and  $\text{NH}_4^+\text{-N}$  were strongly correlated with the first axis, immediately followed by TSS. The temperature was the only parameter which was correlated with the third axis. The axis 1 and axis 2 of PCA with eigenvalues of  $\lambda_1 = 6.78$  and  $\lambda_2 = 2.27$  explained 53.25% of the cumulative variance in phytoplankton- environmental relationship. On the positive side, the first axis is defined by *T. gelatinosa* which is positively correlated with  $\text{PO}_4^{3-}\text{-P}$ ,  $\text{NH}_4^+\text{-N}$  and TSS and negatively with DO, pH, salinity and  $\text{NO}_3^-\text{-N}$ . Cyanophycean like *Anabaena* sp., *Spirulina* sp. and *Anacystis* sp. showed a similar correlation as that of *T. gelatinosa*. Diatoms, such as *Skeletonema* sp. *Chaetoceros* sp. and *Thalassiosira* sp. were



present on the negative side of the first axis and positively correlated with pH, DO, salinity and  $\text{NO}_3^-$ -N.

The multidimensional scaling (MDS) analysis of species abundance data from the Veraval harbour revealed that the significant difference among the stations was spatial rather than temporal (Fig. 10). Based on the MDS analysis the study region was divided into two different groups dominated by *T. gelatinosa* and diatoms respectively. Throughout the study period, the group I comprised of three stations namely V1, V2 and V3 excluding station V1 during 2013. The averaged group I (V1, V2 and V3) similarity was 58 % which is majorly contributed by *T. gelatinosa*. The Group II consisted of near-shore (V4 & V5) and off-shore (V6 & V7) stations from 2013 to 2015 and exhibited 55% similarity. Diatoms, such as *Thalassiosira* sp., *Skeletonema* sp. and *Chaetoceros* sp. dominated this group.

## **Discussion**

The dominance of any species or particular algal composition is a result of a combination of several environmental factors and their adaptation to exploit favourable nutrients more efficiently than others (Reynolds 1998). The *T. gelatinosa* dominated the inner harbour stations throughout the year. The available literature documented *T. gelatinosa* as a cosmopolitan, freshwater form (Gazala et al. 2004; Richter et al. 2014). However, the occurrence of *T. gelatinosa* bloom in the marine environment suggested its adaptation and proliferation in saline water due to favourable nutrients. Since there is lack of river inflow in the harbour region comparatively lower salinity in the inner harbour stations can be due to the addition of sewage flux from the land sources (Mandal et al. 2015). Negative correlation of *T. gelatinosa* with salinity ( $r = -0.97$ ,  $p = <0.0001$ ) explained the optimal growth of *T. gelatinosa* at lower salinity ranges (27.78-34.73) of inner harbour stations rather than near-shore and off-shore stations with relatively higher salinity (33.39-35.98).

## **Factors influencing *T. gelatinosa* growth**

Decreasing spatial trend in *T. gelatinosa* density from inner harbour to off-shore stations and its complete absence at the off-shore station might be due to the augmented level of ammonia in the inner harbour. Due to limited seawater exchange, discharges from fish processing units as well as effluents from sewage plant got accumulated thereby lead to high organic load in the Veraval harbour. Further oxidation of this organic matter has resulted in high ammonia concentration (NIO, 2014 & 2015). Yin and Harrison (2007) also reported the elevated level of ammonia in the Victoria harbour associated with increased sewage disposal. The correlation between the *T. gelatinosa* density and ammonia concentration further tested statistically by applying correlation analysis and Principle

Component Analysis (PCA) which demonstrated a strong positive correlation between *T. gelatinosa* density and ammonia concentration (Table 1 and Fig. 9). Thus the availability of high ammonia at the inner harbour stations is conducive to the proliferation of *T. gelatinosa*. Most algae preferentially utilize ammonia as the nitrogen source over nitrate or nitrite (Gilbert and Goldman 1981; Dortch 1990) as the assimilation of ammonia requires less energy expenditure as against  $\text{NO}_3^-$  which needs to be reduced before it can be used in protein synthesis. The high energy requirement is associated with the assimilation of  $\text{NO}_3^-$  for both inductions of enzyme system and reducing potential (Eppley et al. 1969). Therefore, the energy cost allied with the use of  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$  is likely to play a role in *T. gelatinosa*  $\text{NH}_4^+$  preference. McCarthy et al. (1977) observed that algal species of various taxonomic groups take up ammonia before nitrate when ammonium level is  $>0.5 \mu\text{mol L}^{-1}$ . The increased cell density of *T. gelatinosa* from 2011 to 2015 regardless of variation in ammonia concentration suggested that ammonia was not a limiting nutrient in the Veraval harbour. The experiment carried out by Paasche (1971), Syrett and Morris (1963) and Tam and Wong (1996) with *Dunaliella tertiolecta*, and *Chlorella* sp., respectively suggested that the cells of both species preferred ammonia over nitrate when both N sources were available in the culture medium. From the above discussion, it is clear that ammonia was the principal nitrogen source utilized by *T. gelatinosa* in the Veraval harbour for their growth.

Despite the fact that ammonia is the preferred nitrogen source for phytoplankton growth, it is toxic to cells at elevated concentrations and causes growth inhibition or even cell death (Markou et al. 2014). In the present study, the inner harbour stations recorded high *T. gelatinosa* density with low Chl. *a* concentration. The Chl. *a* : Phae. ratio which serves as a good indicator of the physiological condition of phytoplankton was also found to be  $<1$  at these locations. The elevated concentration of ammonia (av.  $412.9 \mu\text{mol L}^{-1}$ ) hampered the functioning of chlorophyll *a* lead to high phaeophytin (degraded pigment of chlorophyll *a*) concentration (Nagengast et al. 2014; Markou et al. 2016 and Dean 2017). The study made by Drath et al. 2008 demonstrated that ammonia toxicity increases the sensitivity of PS II to photodamage. Therefore, from the study, it could be understood that the ammonia toxicity could be the major reason for *T. gelatinosa* cell death and thereby increase phaeophytin content in the Veraval harbour.  $\text{PO}_4^{3-}\text{-P}$  also exhibited high concentration (av. 32.98) in the inner harbour region. The elevated concentration of  $\text{PO}_4^{3-}\text{-P}$  was almost identical to the pattern observed for ammonia. Further, ammonia and phosphate displayed a positive correlation ( $r = 0.76$ ,  $p < 0.0001$ ) indicating disposal of sewage and sediment release during anoxia/hypoxia period could be an important source for  $\text{PO}_4^{3-}\text{-P}$  (Howarth et al. 2011). In support DO varied negatively with  $\text{NH}_4^+\text{-N}$  ( $r = -0.72$ ,  $p = <0.0001$ ) and  $\text{PO}_4^{3-}\text{-P}$  ( $r = -0.97$ ,  $p = <0.0001$ ). A significant positive correlation

between *T. gelatinosa* density and  $\text{PO}_4^{3-}\text{-P}$  ( $r = 0.77$ ,  $p < 0.0001$ ) also showed its growth requirements. Maneeruttanarungroj et al. 2010 carried out experiment with seven different types of culture medium and reported the fastest cell growth of *T. gelatinosa* in TAP (tris-acetate-phosphate) medium. Ranjan et al. 2007 also explained the positive relation between phosphate concentration and chlorophytes. The anoxic condition in Veraval harbour suppressed further oxidation of  $\text{NH}_4^+\text{-N}$  which resulted in low concentrations of  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  in the water column. Since  $\text{NH}_4^+\text{-N}$  is the preferred nitrogen source for *T. gelatinosa* growth a negative correlation was found with  $\text{NO}_3^-\text{-N}$  ( $r = 0.73$ ,  $p < 0.0001$ ).

Habitat ecology of *T. gelatinosa* and their relationship with environmental drivers is inadequately documented in the previously cited literatures. The studies conducted in Antarctica (Vinocur and Pizarro 2000); Germany (Gutowski et al. 2004) and Arctic (Richter et al. 2014) are the few which provides the information on basic nutrients. Vinocur and Pizarro 2000 studied the microbial mat of twenty six lakes of Antarctica in which occurrence of *T. gelatinosa* was associated with high ammonia ( $13\text{-}29 \mu\text{g L}^{-1}$ ) and phosphate ( $6\text{-}41 \mu\text{g L}^{-1}$ ) content rather than nitrate ( $0\text{-}19 \mu\text{g L}^{-1}$ ) concentration in the water column. Gutowski et al. 2004 also reported the presence of *T. gelatinosa* from the fresh water habitat where the nitrate ( $0.1\text{-}11.8 \text{mg L}^{-1}$ ) and ammonia ( $0.01\text{-}7.99 \text{mg L}^{-1}$ ) reported in elevated condition. In their results, occurrence of *T. gelatinosa* was found in comparatively high ammonia content than nitrate. The present study observation was identical with the studies made by Vinocur and Pizarro 2000 and Gutowski et al. 2004 in Antarctica and Germany respectively. Although the ammonia and phosphate were promoting the growth of *T. gelatinosa* in these two ecosystems (Antarctic and Germany) it never lead to a bloom condition.

In the study region, pH was significantly correlated with the *T. gelatinosa* density (Table 1). However, it is difficult to know whether the low pH at the harbour stations is the cause or the result of *T. gelatinosa* bloom. Lower values of pH at the inner harbour stations can be due to the environmental stress associated with the release of effluents from various sources in the harbour area and decomposition of anthropogenic organic matter under anoxic/hypoxic conditions of the harbour (NIO 2014, 2015; Majithiya et al. 2017).

### ***Tetraspora gelatinosa* bloom and hypoxia**

Coastal hypoxia/anoxia is believed to be largely associated with anthropogenic eutrophication (Ram et al. 2014; Wang et al. 2017) and it is increasing at the rate of 5% per year throughout the world. Hypoxia ( $\text{DO} < 2 \text{mg L}^{-1}$ ) and anoxia ( $\text{DO} < 0.2 \text{mg L}^{-1}$ ) can alter many ecological processes such as

nutrient biogeochemical cycle (Testa and Kemp 2012; Wright et al. 2012), redox environment in sediment (Middelburg and Levin 2009) and enhance coastal acidification (Feely et al. 2010).

In the study region, inner harbour stations are encountered with low DO, high turbidity and elevated level of ammonia thus affecting the phytoplankton growth. Regardless of the season, the harbour receives around 20,000 m<sup>3</sup> d<sup>-1</sup> of anthropogenic waste from fish processing industries and about 20,000 m<sup>3</sup> d<sup>-1</sup> of sewage from Veraval city (CPCB, 2009-2010). Photochemical oxidation, microbial degradation and flocculation are the major processes which removes the organic matter from the coastal region (Wang et al. 2004). Veraval harbour is a semi-enclosed coastal area with stagnant water condition. Construction of jetty perpendicular to the water flow, lack of turbulence, wind action, tidal effect and siltation at mouth makes dilution of organic matter entering the harbour is an impossible task. Therefore, the elevated level of organic matter (from land based activities and from *T. gelatinosa* bloom fuelled by nutrient loading) induced hypoxia/anoxia throughout the water column in the Veraval harbour (Majiyhiya et al. 2017). Paerl et al. 1998 reported the organic matter enrichment associated with land runoff causes the hypoxia/anoxia throughout the water column.

In addition to this, inner harbour also faces high turbidity (61.1-230.5 mg L<sup>-1</sup>; av. 121.9 mg L<sup>-1</sup>) leading to less evolution of oxygen through photosynthetic activity. Reduction in light penetration as a consequence of increased turbidity causes a transition in the chloroplast from light II state to light I state. The shift in the light state results in decreased oxygen evolution as the proportion of red light increases with decreasing light intensity (Folkowski and Owens 1978). Anoxia can have adverse effects on marine organisms such as the slower growth rate, elevated stress level and impairment in productivity. It also triggers mortality events, resulting in depletion of living organisms in the ecosystem which eventually lead to “dead zones” (Ram et al. 2014).

### **Phytoplankton diversity index**

Biodiversity is the key ecological factor related to the regulation and functioning of the ecosystem. Greater ecological diversity is related to the stability and sustainability of the ecosystem. It also buffers the ecosystem against environmental fluctuations (Chalar 2009). Shannon - Wiener diversity index ( $H'$ ) is one of the most widely used diversity indices for measuring phytoplankton diversity. It can be used as a proxy to define ecosystem under pollution stress or Eutrophication (Balloch et al. 1976).

In the Veraval harbour, *T. gelatinosa* abundance has resulted in very low diversity index (0.01-0.55; av. 0.25) in the inner harbour stations. Allelopathic effect of *T. gelatinosa* might be one of the reasons for low diversity at inner harbour stations. The study carried out by Graneli et al. (2008)

showed that the bloom-forming species secrete allelochemicals which inhibit the growth of co-occurring species. Gao and Song (2005) found that single species dominance can also decrease the diversity to a minimal level. In a healthy environment, the Shannon- Wiener diversity index is in the range of 2 – 4 (Gao and song 2005). Applying these criteria to phytoplankton diversity, we can assume that the Veraval harbour is a polluted environment as the diversity ranged from 0.01 to 1.57 throughout the study period.

Further, the near-shore, as well as off-shore stations, recorded high diversity index (0.03-3.95; av. 2.17) because of less anthropogenic effect in this region (Fig. 8). *Thalassiosira* sp. *Skeletonema* sp. and *Chaetoceros* sp. dominated the near-shore and off-shore stations which were governed by  $\text{NO}_3^-$  and salinity (Figs. 4 and 9). Diatoms appear to be  $\text{NO}_3^-$  opportunistic; they are the dominant protist found in  $\text{NO}_3^-$  rich water column (Glibert et al. 2016). The distinct salinity and  $\text{NO}_3^-$  preference by these species was further shown by Achary et al. (2014) reported the dominance of these diatoms associated with salinity and  $\text{NO}_2+\text{NO}_3$  content of the water column.

## **Conclusion**

The present study is the first endeavour reporting the *T. gelatinosa* bloom and the environmental drivers for proliferation in the marine environment. Morphological (SEM) and genetic analysis confirm its identity as *T. gelatinosa* (RKHDKY055012). Elevated level of organic matter derived from sewage disposal as well as *T. gelatinosa* bloom induced hypoxia/anoxia throughout the water column in the Veraval harbour. Ammonia the principal nitrogen source derived from sewage disposal was the main causative factor for *T. gelatinosa* bloom. At the same time elevated level of ammonia (av.  $412.9 \mu\text{mol L}^{-1}$ ) hampered the chlorophyll *a* functioning, that lead to high phaeophytin (degraded pigment of chlorophyll *a*) concentration. Moreover, reduction in light penetration due to high turbidity caused the degradation of chlorophyll *a* into phaeophytin giving rise to low chl. *a* : phae. ratio. Besides this, due to less ammonia concentration in the off-shore waters the *T. gelatinosa* density decreased from inner harbour to off-shore stations which ultimately lead to increase in the diversity index in the off-shore waters. However, further study may be required to understand the genetic modification allowing *T. gelatinosa* to propagate in a marine as well as in anoxic/hypoxic environment for its optimum growth.

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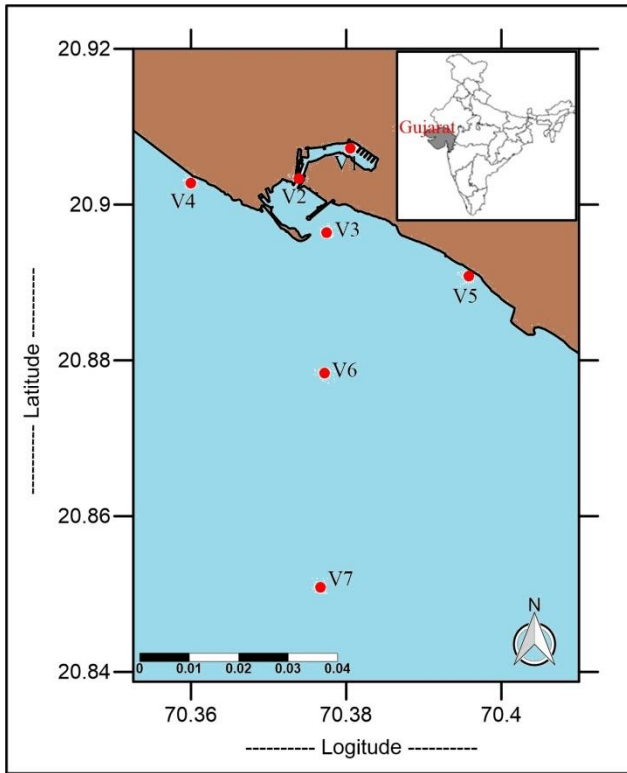
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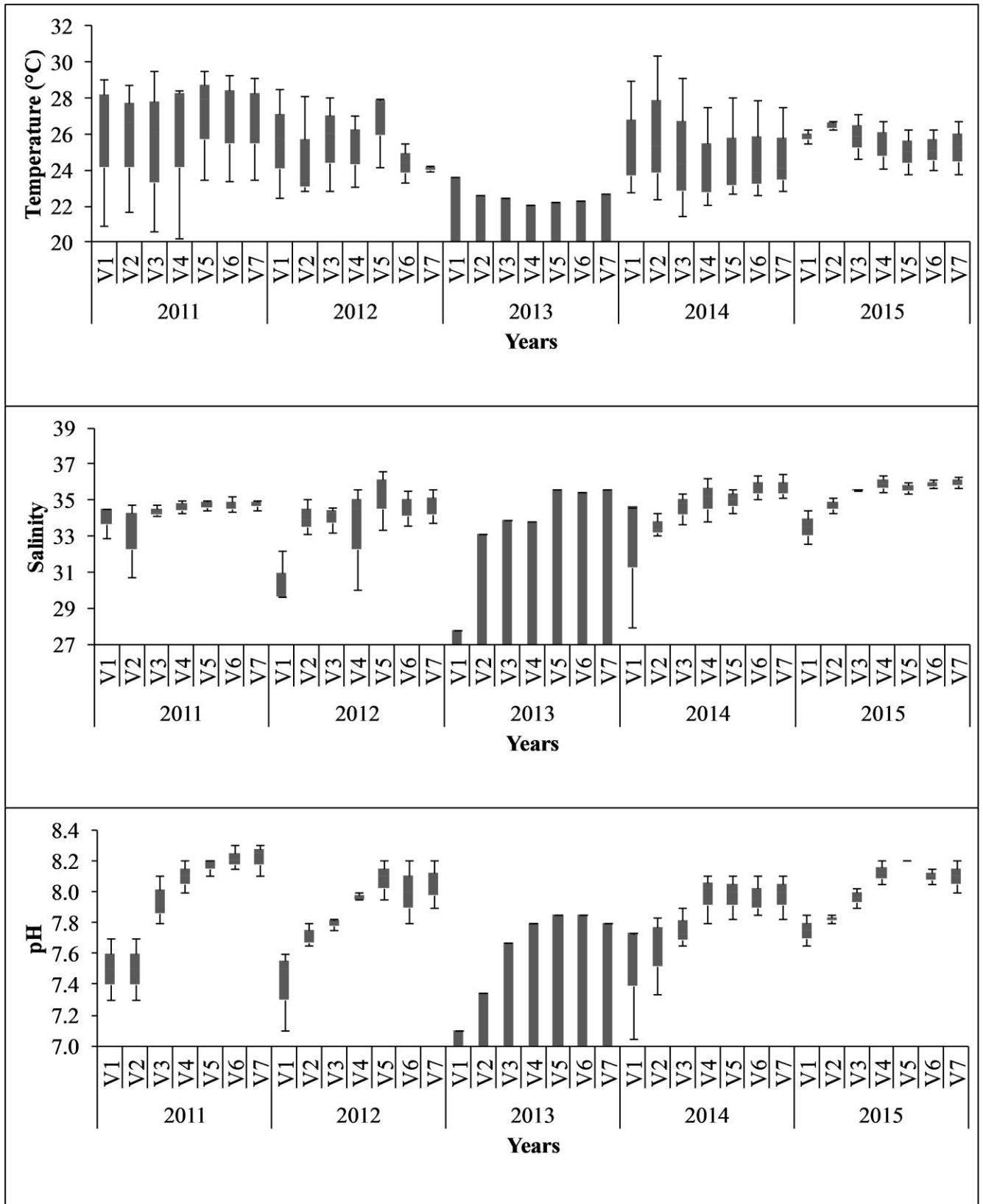
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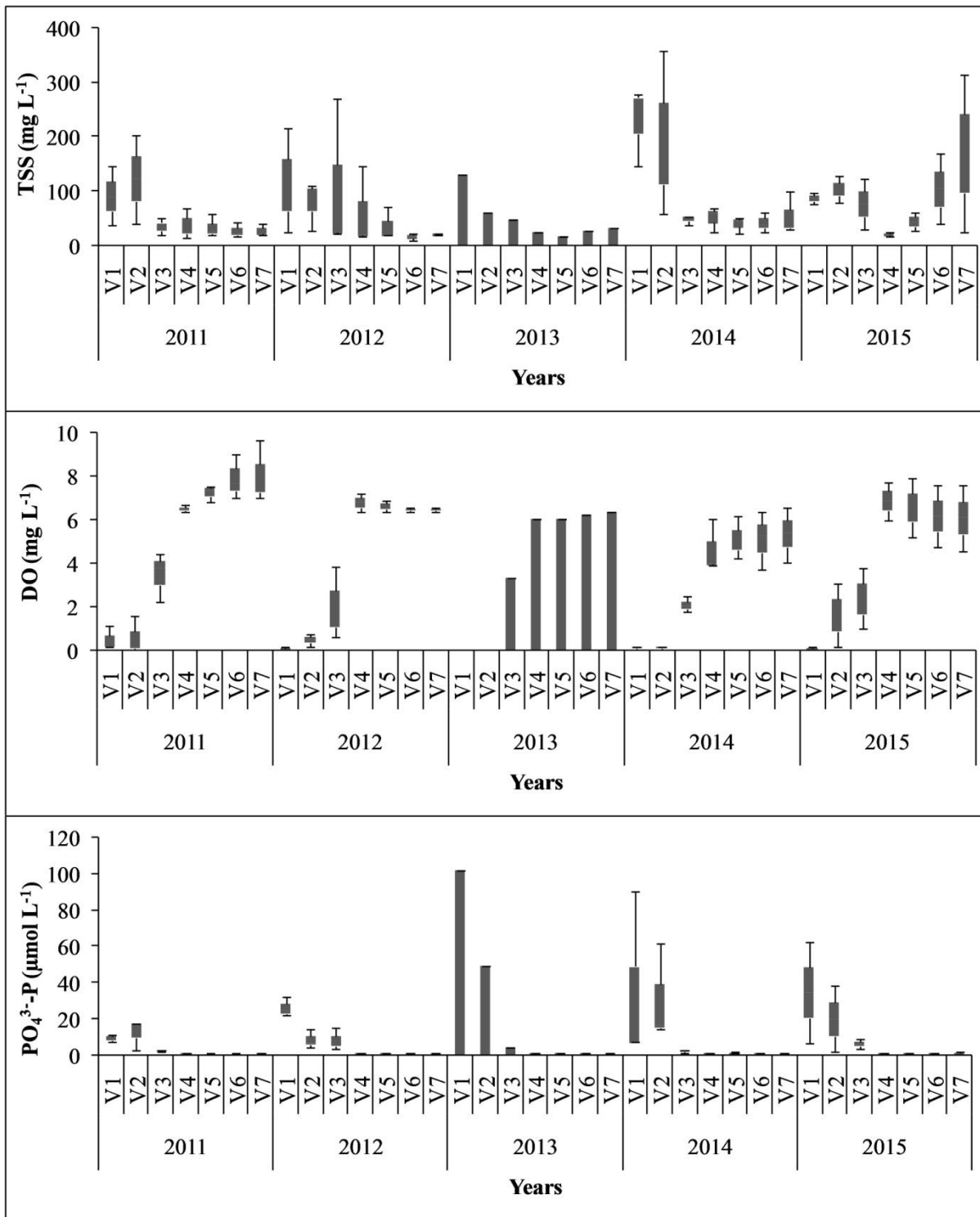


**Fig. 1** Location and sampling sites off Veraval



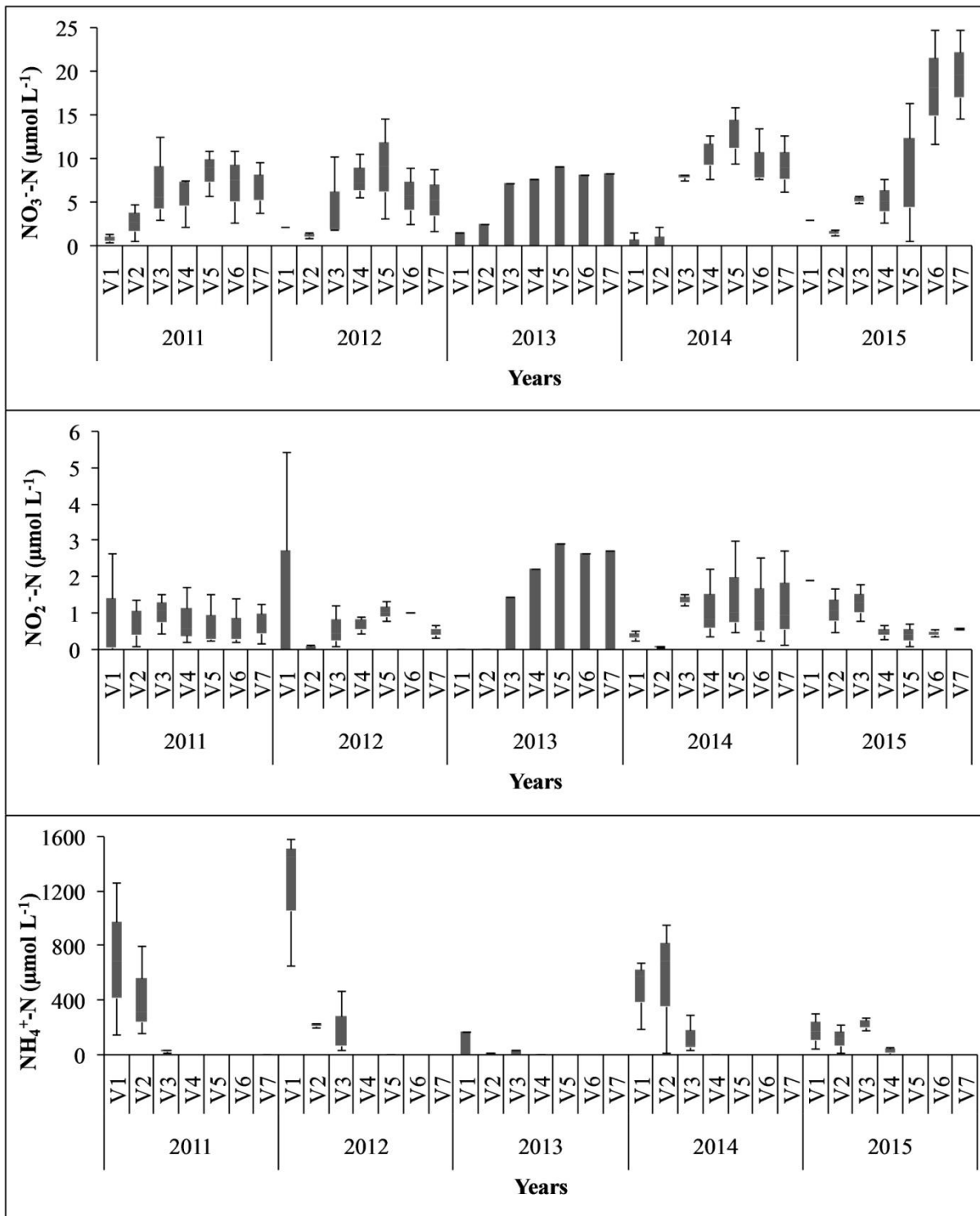
**Fig. 2** Spatio-temporal variation in physico-chemical parameters (temperature, pH and salinity) from the Veraval harbour during 2011-2015.

**Fig. 2** Continue 1

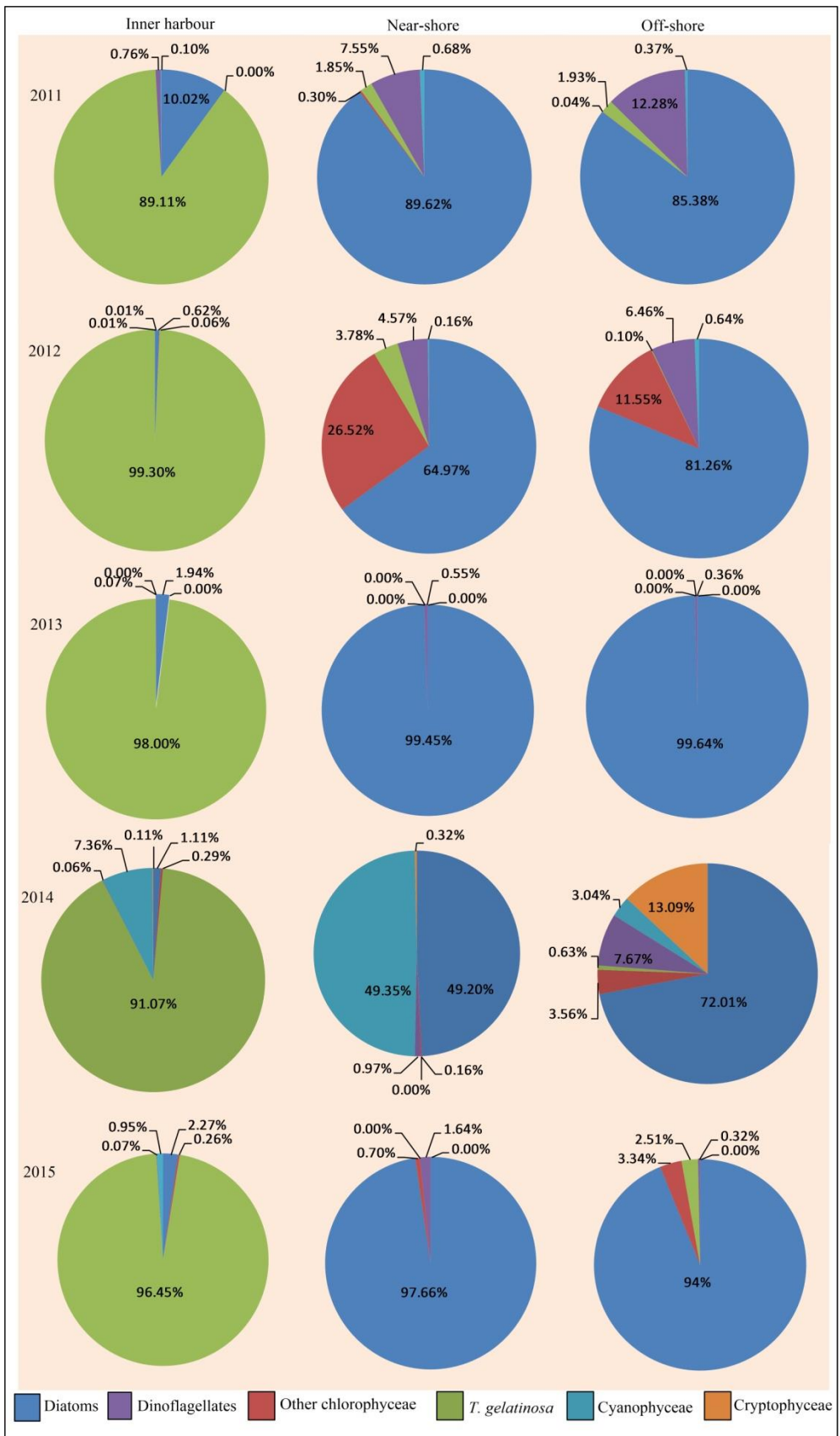


**Fig. 2** Spatio-temporal variation in physico-chemical parameters (TSS, DO and PO<sub>4</sub><sup>3-</sup>) from the Veraval harbour during 2011-2015.

**Fig. 2** Continue 2

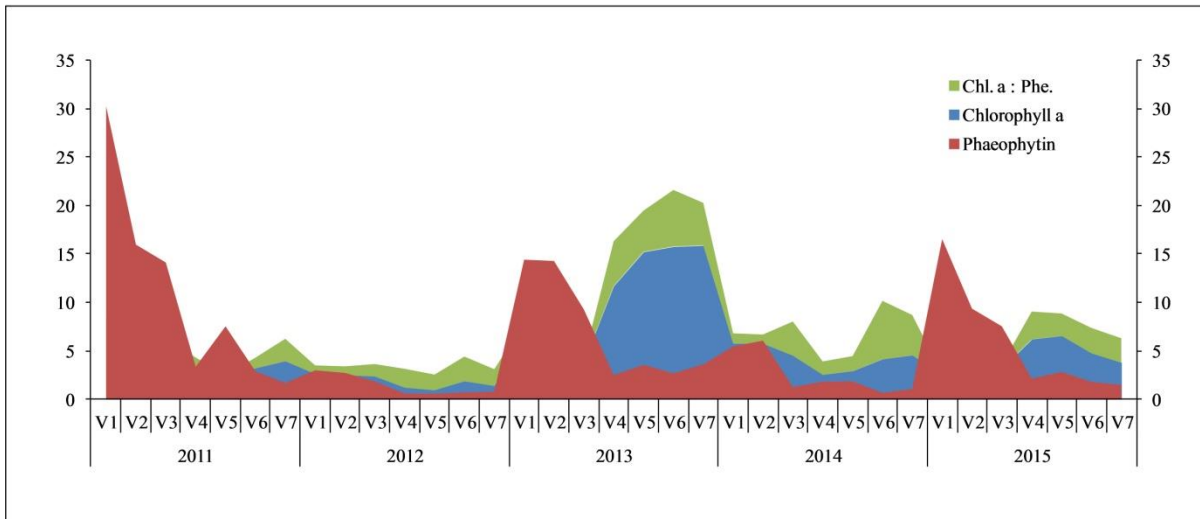


**Fig. 2** Spatio-temporal variation in physico-chemical parameters ( $\text{NO}_3$ ,  $\text{NO}_2$  and  $\text{NH}_4$ ) from the Veraval harbour during 2011-2015.

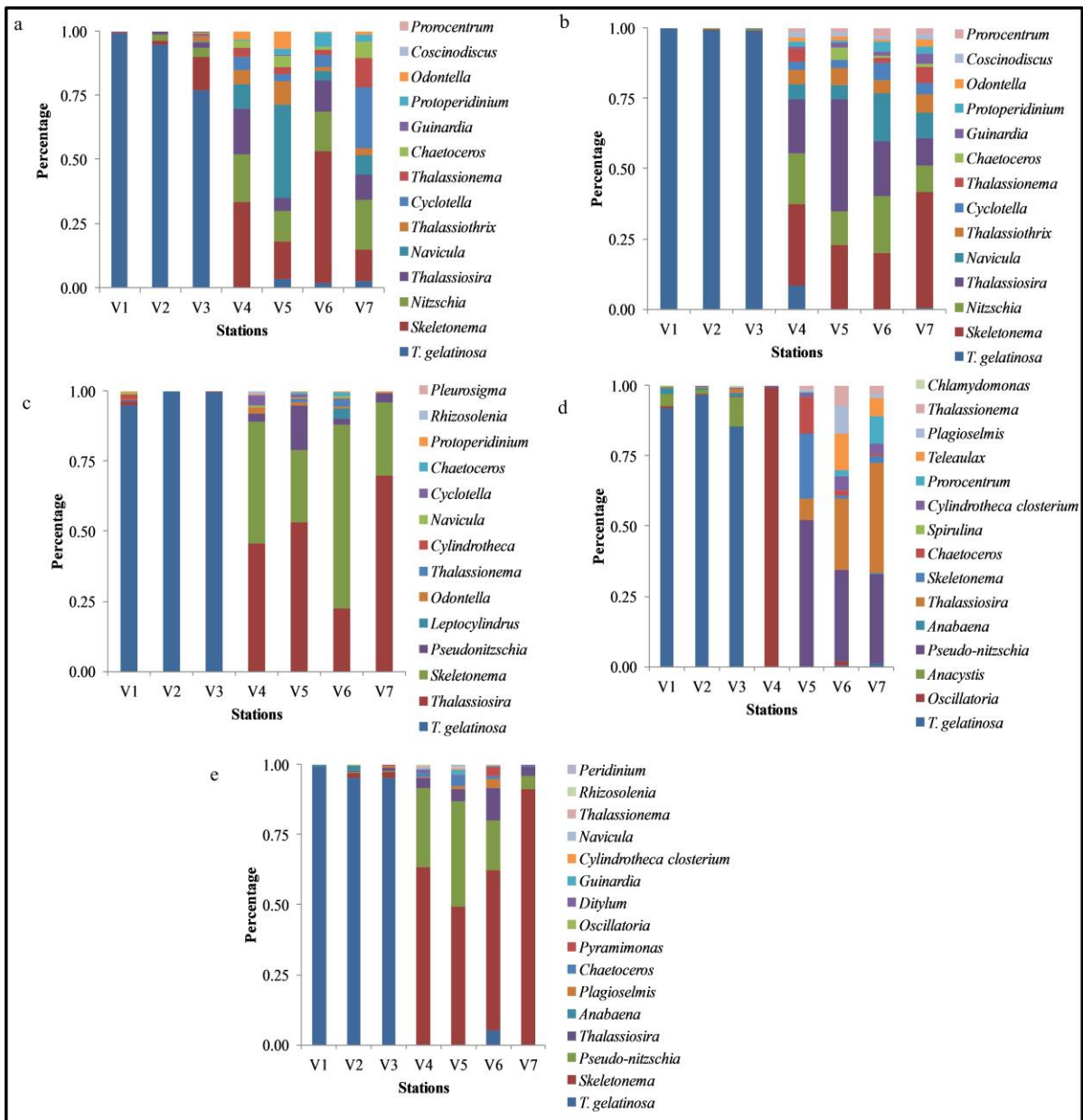


**Fig. 3** Spatio-temporal distribution of phytoplankton community structure from 2011 to 2015 in Veraval harbour.

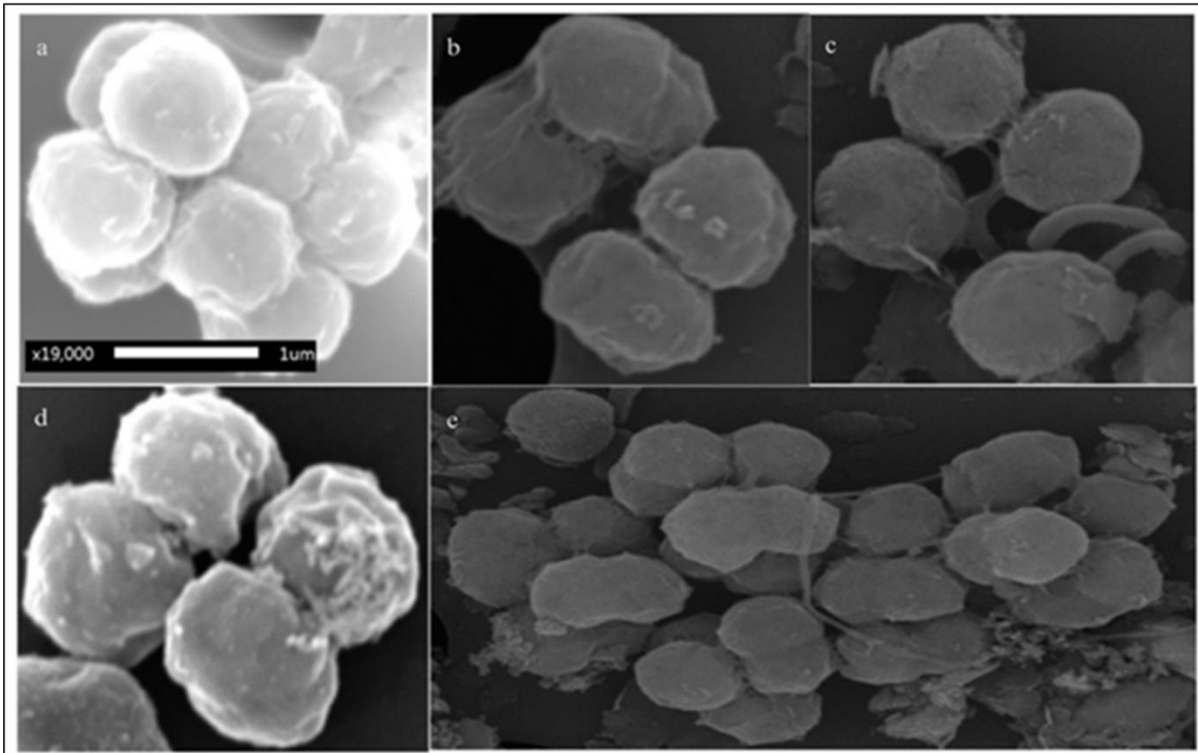




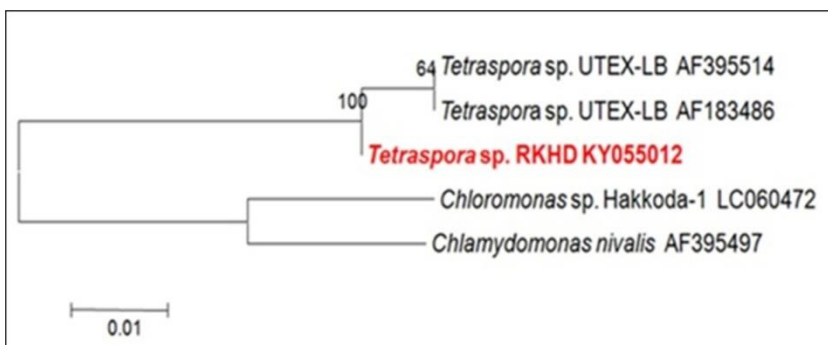
**Fig. 4** Spatio-temporal distribution of Chlorophyll *a* (mg m<sup>-3</sup>) (Primary axis), Phaeophytin (mg m<sup>-3</sup>) (secondary axis) and Chlorophyll *a* : Phaeophytin ratio (Chl. *a* : Phe.) (Primary axis) from Veraval during 2011-2015.



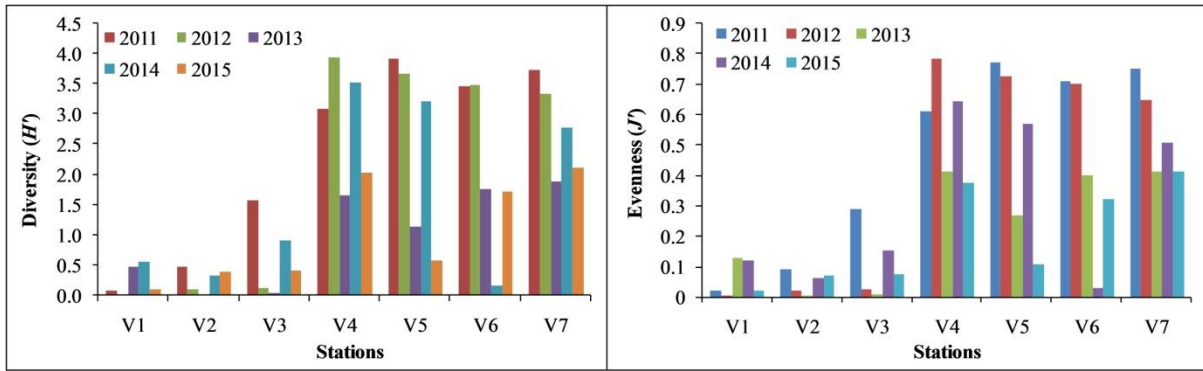
**Fig. 5** Spatio-temporal variation in relative abundance of dominant phytoplankton genera (a - 2011, b - 2012, c - 2013, d - 2014, e - 2015) in Veraval harbour.



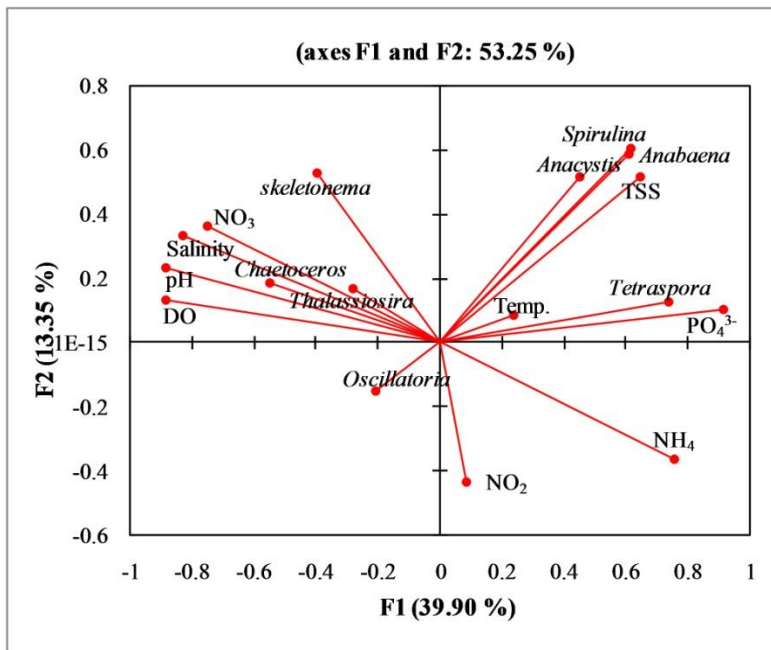
**Fig. 6** Scanning electron microscopy (SEM) of *T. gelatinosa* cells collected from Veraval harbour, Gujarat (a, c, d – Tetrad arrangement, b – disruption of the mucilaginous sheath, e – enlarge part of the colony).



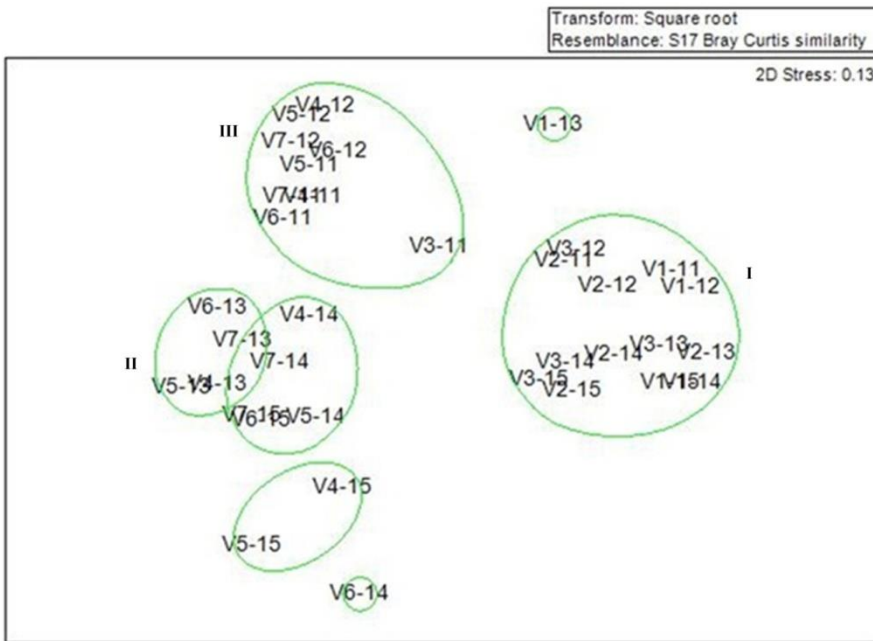
**Fig. 7** Phylogenetic tree of *Tetrastroma* RKHB based on 28S rRNA gene sequence analysis, constructed through neighbour-joining method with bootstrap values as a percentage at the nodes.



**Fig. 8** Spatio-temporal variation in phytoplankton diversity ( $H'$ ) and evenness ( $J'$ ) in the Veraval harbour from 2011-2015.



**Fig. 9** PCA bi-plot of species environmental relationship



**Fig. 10** Multidimensional scaling (MDS) ordination of the fourth root transformed species data at each site sampled during 2011 to 2015 in Veraval harbour

**Table 1** Summary of the results of correlation analysis. Values marked in bold are significant

Variables	Temp.	TSS	pH	Salinity	DO	NO <sub>2</sub> <sup>-</sup> -N	NO <sub>3</sub> <sup>-</sup> -N	NH <sub>4</sub> <sup>+</sup> -N	PO <sub>4</sub> <sup>3-</sup> -P	Tetra. Density	Total Density
Temp.	<b>1.00</b>										
TSS	0.29	<b>1.00</b>									
pH	0.24	<b>-0.54**</b>	<b>1.00</b>								
Salinity	-0.05	<b>-0.40*</b>	<b>0.72***</b>	<b>1.00</b>							
DO	0.10	<b>-0.70***</b>	<b>0.89***</b>	<b>0.60***</b>	<b>1.00</b>						
NO <sub>2</sub> <sup>-</sup> -N	-0.24	<b>-0.36*</b>	-0.06	0.18	0.05	<b>1.00</b>					
NO <sub>3</sub> <sup>-</sup> -N	-0.28	<b>-0.39*</b>	<b>0.60***</b>	<b>0.72***</b>	<b>0.56***</b>	<b>0.37*</b>	<b>1.00</b>				
NH <sub>4</sub> <sup>+</sup> -N	0.20	<b>0.54**</b>	<b>-0.75***</b>	<b>-0.67***</b>	<b>-0.72***</b>	-0.10	<b>-0.78***</b>	<b>1.00</b>			
PO <sub>4</sub> <sup>3-</sup> -P	-0.08	<b>0.66***</b>	<b>-0.87***</b>	<b>-0.64***</b>	<b>-0.97***</b>	-0.06	<b>-0.63***</b>	<b>0.76***</b>	<b>1.00</b>		
Tetra. Density	0.28	<b>0.53**</b>	<b>-0.63***</b>	<b>-0.62***</b>	<b>-0.72***</b>	-0.14	<b>-0.73***</b>	<b>0.82***</b>	<b>0.77***</b>	<b>1.00</b>	
Total Density	-0.01	<b>0.53**</b>	<b>-0.53**</b>	-0.21	<b>-0.65***</b>	-0.03	<b>-0.39*</b>	<b>0.53**</b>	<b>0.61***</b>	<b>0.61***</b>	<b>1.00</b>

\* $p \leq 0.05$ ; \*\* $p \leq 0.001$ ; \*\*\* $p \leq 0.0001$ ;  $N$  (no. of samples) = 35

**Table 2** PCA results of species environmental relationship in Veraval Harbour

<b>Variables</b>	<b>F1</b>	<b>F2</b>	<b>F3</b>	<b>F4</b>
Temp.	0.24	0.09	<b>0.71</b>	0.09
TSS	<b>0.65</b>	0.51	-0.05	0.43
pH	<b>-0.89</b>	0.23	0.14	-0.13
Salinity	<b>-0.83</b>	0.33	0.08	-0.14
DO	<b>-0.88</b>	0.13	-0.08	-0.04
NO <sub>2</sub> <sup>-</sup> -N	0.09	-0.43	0.40	<b>-0.49</b>
NO <sub>3</sub> <sup>-</sup> -N	<b>-0.75</b>	0.36	-0.01	0.18
NH <sub>4</sub> <sup>+</sup> -N	<b>0.76</b>	-0.36	-0.02	0.35
PO <sub>4</sub> <sup>3-</sup> -P	<b>0.91</b>	0.10	0.17	0.03
<i>Anabaena</i>	<b>0.61</b>	0.59	0.14	-0.34
<i>Anacystis</i>	0.45	<b>0.52</b>	-0.34	-0.39
<i>Chaetoceros</i>	<b>-0.55</b>	0.18	0.16	0.04
<i>Oscillatoria</i>	-0.21	-0.15	-0.38	-0.39
<i>Skeletonema</i>	-0.39	<b>0.52</b>	0.28	0.40
<i>Spirulina</i>	<b>0.61</b>	0.61	-0.32	-0.16
<i>T. gelatinosa</i>	<b>0.74</b>	0.13	0.42	-0.24
<i>Thalassiosira</i>	-0.28	0.17	0.42	-0.29
Eigenvalue	6.78	2.27	1.56	1.38
Variability (%)	39.90	13.35	9.16	8.15
Cumulative %	39.90	53.25	62.41	70.56