2.1 Coastal and estuarine stations along the southwest coast of India

Coastal and estuarine samplings on seasonal basis (pre-monsoon, monsoon and post-monsoon) from selected stations (Map 1) and continuous monitoring for algal blooms, along the southwest coast of India were carried out for a period of two years (2009-2011). The selection of stations was made on the basis of the previous occurrence of algal blooms. The details of the stations are given in Table 1. Among the stations, three are coastal (Azheekode, Kodikkal and Puthiyangadi) and the rest are estuarine (Balathuruth, Mahe and Thykadapuram). Sampling has been carried out for the study of bacteria associated with algal blooms, analysis of qualitative and quantitative distribution of microalgae and hydrographical variables. The terms PRM, MON and POM are used to represent pre-monsoon, monsoon and post-monsoon, respectively.
2.1.1 Stations

Map 1
Local stations along the Kerala coast
Table 1 Coastal and estuarine station locations.

<table>
<thead>
<tr>
<th>Stations</th>
<th>Name of the Stations</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azheekode (Thrissur)</td>
<td>10° 11’ 02” N</td>
<td>76° 09’ 22” E</td>
</tr>
<tr>
<td>2</td>
<td>Balathuruth (Kozhikode) -E*</td>
<td>11° 07’ 50” N</td>
<td>75° 49’ 57” E</td>
</tr>
<tr>
<td>3</td>
<td>Kodikkal (Kozhikode)</td>
<td>11° 28’ 43” N</td>
<td>75° 36’ 10” E</td>
</tr>
<tr>
<td>4</td>
<td>Mahe -E*</td>
<td>11° 42’ 18” N</td>
<td>75° 32’ 36” E</td>
</tr>
<tr>
<td>5</td>
<td>Puthiyangadi (Kannur)</td>
<td>12° 01’ 11” N</td>
<td>75° 21’ 89” E</td>
</tr>
<tr>
<td>6</td>
<td>Thykadapuram (Kasaragod) -E*</td>
<td>12° 12’ 39” N</td>
<td>75° 07’ 33” E</td>
</tr>
</tbody>
</table>

*E* stands for estuarine stations and the others are coastal stations.

Besides regular sampling from these stations, sampling has been carried out from Bekalam* (Kasaragod) (Lat. 12° 38’ 02” N & Long. 75° 04’ 31” E) during a bloom event from 10th to 12th October 2009.

2.1.2 Hydrographic parameters.

Hydrographic parameters such as temperature, salinity and pH were measured soon after the collection of surface water sample.

2.1.2.1 Temperature

Temperature measurement in °C (degree Celsius) was done by using a precision mercury thermometer with an accuracy ± 0.01°C.

2.1.2.2 Salinity

Salinity in psu (practical salinity unit) was measured using a hand held refractometer (Erma- Japan).

2.1.2.3 pH

pH measurement was done using a portable pH meter (Eutech eco Tester pH2) with an accuracy ± 0.01.
2.1.2.4 Dissolved Oxygen

Dissolved oxygen was determined by Winkler’s (1888) method, for which water sample was collected in 50 ml ground stoppered Biological Oxygen Demand (BOD) bottle, fixed with Winkler A (Manganese Sulphate) and Winkler B (alkaline Potassium Iodide) solution.

2.1.2.5 Nutrient analysis

The quantitative estimation of major nutrients like nitrate, nitrite, silicate and phosphate were carried out in the laboratory according to the methods of Strickland and Parsons (1972) and Fischer and Zhang (2006).

2.1.2.6 Primary Productivity

Light and dark bottle method (Gaarder and Gran, 1927) was used for the estimation of primary productivity. For the analysis of primary production rate, a set of three BOD bottles of 50 ml capacity was used, in which one was taken as initial bottle or control bottle (IB), the second one as light bottle (LB) and the third one as dark bottle (DB). Surface water sample was collected by using a clean bucket and the sample was siphoned into the bottle by using a clean polythene tube. One end of the polythene tube was fitted with bolting silk, which measures about 200 µm, in order to eliminate the presence of zooplankters in the siphoned sample, which will interfere with the oxygen content in the experiment bottles. Care was taken to avoid the agitation of water. While filling the samples into the bottles, it was made sure that the polythene tube touched the bottom of the bottles and all the bottles were properly stoppered without trapping air bubbles inside the bottles. All these measures were taken to avoid the formation of air bubbles, which interfere with the oxygen content of the sample.
Materials and Methods

The initial bottle sample was immediately fixed with 0.5 ml of manganese sulphate (Winkler A) and 0.5 ml of alkaline potassium iodide (Winkler B). The dark bottle was wrapped with aluminum foil and kept in a black polythene bag in order to avoid the interference of sunlight. Both dark and light bottles were incubated in a transparent acrylic chamber for three hours. After three hour incubation the light and dark bottles were fixed with the Winkler reagents and the oxygen content of all the three bottles (LB, DB and IB) was determined by Winkler’s chemical titration method.

The resultant values obtained were generalized for day/hours and the primary productivity was calculated by using the calculations described below. 1.25 was taken as the photosynthetic quotient (PQ) (Gaarder and Gran, 1927).

Calculations

Gross production = O₂ content of light bottle - O₂ content of dark bottle ------ A
Net production = O₂ content of light bottle - O₂ content of control bottle ----- B
Respiration = O₂ content of control bottle - O₂ content of dark bottle --------- C

The period of incubation was three hours, then

Gross production (mgC/L/hr) = A × 0.375/PQ × 3 ------------------------ D
Net Production (mgC/L/hr) = B × 0.375/PQ × 3 -------------------------- E
Gross or net production (mgC/L/day) = D or E × 12 --------------------- F
Gross or net production (gC/m³/day) = F × 1000 ×1000
2.1.2.7 Estimation of Pigments

2.1.2.7.1 Estimation of Chlorophyll

For the chlorophyll estimation, surface water sample was collected by using a clean plastic bucket and transferred into a one litre clean black plastic can and stored in a frozen container until further analysis was carried out in the laboratory. The pigments chlorophyll \(a, b, c\) and carotenoids were extracted by using 90% acetone.

The surface water sample, free of zooplankters was filtered through GF/C glass filter paper having a pore size of 0.45 µm. Before filtering, 1 ml of 1% magnesium carbonate \((\text{MgCO}_3)\) was added into the glass filter paper in order to avoid the development of acidity and thereby the gradual degradation of the pigment in the extract. A low suction was applied on the glass filter paper to allow the sucking out of the \(\text{MgCO}_3\) as a thin layer. After the formation of a thin bed of \(\text{MgCO}_3\) on the filter paper, one litre of water sample was filtered through the filtration apparatus fitted with a vacuum pump (Millipore, USA).

The filter paper was then placed in an acid free screw capped test tube containing 10 ml of 90% acetone and kept in a refrigerator for 24 hours incubation. After incubation the filter papers were taken out and ground well with a clean glass rod in order to accelerate the extraction of pigments and transferred it into a centrifuge tube and centrifuged for 10 minutes at 3500 rpm. The clear supernatant was made up to 10 ml by adding fresh 90% acetone. The absorbance of coloured acetone extract was measured by using a UV-Vis Spectrophotometer (Hitachi U-3900) against 90% acetone as blank at multiple wavelengths of 750, 665, 645, 630 and 450 nm, which are considered as the
maximum absorption wavelength of the pigments. The correction of all the extinction values for a small turbidity blank were done by subtracting the optical density of 750 nm from the 665, 645 and 630 nm absorptions (Strickland and Parsons, 1972).

The equation used to find out the chlorophyll contents are described below.

\[
\text{Chlorophyll } a (C_a) = 11.85 \times E_{665} - 1.54 \times E_{645} - 0.08 \times E_{630}
\]

\[
\text{Chlorophyll } b (C_b) = 21.03 \times E_{645} - 5.43 \times E_{665} - 2.66 \times E_{630}
\]

\[
\text{Chlorophyll } c (C_c) = 24.52 \times E_{630} - 1.67 \times E_{665} - 7.60 \times E_{645}
\]

...where, ‘E’ is the absorbance at different wavelengths in the respective wavelengths.

\[
\text{Chlorophyll } a (\mu g/L) = \frac{C_a \times V}{V \times I}
\]

\[
\text{Chlorophyll } b (\mu g/L) = \frac{C_b \times V}{V \times I}
\]

\[
\text{Chlorophyll } c (\mu g/L) = \frac{C_c \times V}{V \times I}
\]

2.1.2.7.2 Estimation of Carotenoids

For the analysis of carotenoids the above procedure was followed and the spectrophotometric measurement was done at wavelengths of 510 and 480 nm.

\[
\text{Carotenoids } (C_P) \mu g/ L = 7.6 [(E_{480}-E_{750}) - (1.49 \times E_{510}-E_{750})]
\]

\[
= \frac{C_P \times V}{V \times I}
\]
where, ‘v’ is volume of acetone (ml), ‘V’ is volume of water (L) filtered for extraction and ‘l’ is the path length (cm) of cuvette used in spectrophotometer.

2.1.2.8 Phytoplankton Analysis

For the qualitative and quantitative analysis, microalgae were filtered from 50 litre of surface water by using 20 µm bolting silk and the filtered sample was preserved in 2-3% neutralised formaldehyde/Lugol’s iodine. The quantitative estimation was carried out by using Sedgewick-Rafter counting cell.

The microalgal identification was done based on the standard keys (Allen and Cupp, 1935; Venkataraman, 1939; Cupp, 1943; Subrahmanyan, 1946; Hustedt, 1955; Desikachary, 1959; Hendey, 1964; Simonsen, 1974; Gopinathan, 1984; Jin Dexiang et al., 1985; Desikachary and Sreelatha, 1989; Hallegraeff et al., 1995; Tomas, 1997; Karlson et al., 2010).

2.1.3 Sample preparation for Environmental Scanning Electron Microscopy (ESEM)

The algal cells were treated with 0.5% glutaraldehyde prepared in distilled water and kept for 30 minutes to clean the cells thoroughly. The cells were washed with distilled water by centrifuging gently (500 rpm for 2 minutes) for three times to remove the glutaraldehyde as well as the salt content completely. The sample was then air dried over a clean glass slide. Care was taken to avoid breakage of the weakly silicified algal cells. The images of the prepared sample were taken from the Environmental Scanning Electron Microscope (Carl Zeiss EVO-18) at National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum.
2.1.4 Isolation of bloom associated bacteria

2.1.4.1 Sampling, bacterial isolation and identification

Isolation of bloom associated bacteria was done as described by Croci et al. (2006) with some modifications. The bloom sample was filtered through 1 µm GF/C filter paper by using a sterile vacuum filtration unit to retain the phytoplankton cells. Algal cell mass with attached bacterial cells remained in the filter paper and the cells were repeatedly washed with sterile sea water in order to separate out the freely associated bacteria completely. Centrifugation (5000 rpm for 2 minutes) was carried out to isolate the loosely attached bacteria in the algal cells. The centrifuged algal cell pellets were sonicated (70 amplitude for 1 minute) by using an Ultrasonicator (Sonics and Materials Inc., USA) in order to isolate the firmly associated bacteria. The treated algal sample was serially diluted in sterile sea water and plated on to ZoBell 2216e marine agar and nutrient agar (HiMedia, India) media by standard plate count method and incubated at 28 ± 2°C for 48-72 hours to estimate the Total Heterotrophic Bacteria (THB) present in the sample. The individual bacterial colonies that developed were isolated and purified. The purified isolates were identified up to generic level based on cell morphology and biochemical reactions as per Bergey’s Manual of Determinative Bacteriology (2000) (Appendix II). THB associated with reference sample was also estimated by standard plate count method.

2.1.4.2 Tests used for the identification of bacteria

2.1.4.2.1 Gram staining

For staining, smears were prepared on clean glass slides using 12-18 hours old bacterial cultures. The primary stain, ammonium oxalate crystal violet was
added to the fixed smear and allowed to stand for one minute. Then the slides were rinsed gently in running water. Then Gram’s iodine solution was added as a mordant and allowed to stand for one minute. The slides were washed and treated with acetone (decolourizer) for 30 seconds. After washing the smear, the counter stain safranin-O was added and allowed to stand for one minute. The smear was rinsed, allowed to air dry and observed under oil immersion objective lens of a microscope. Gram positive bacteria appeared in violet or purple color, whereas gram negatives were pink in colour.

2.1.3.4.2 Spore staining

Gram positive strains were subjected to spore staining. Smears were prepared using 60-72 hours old cultures. The slides were flooded with malachite green and allowed to react at room temperature for one minute. Then the slides were periodically heated by using a Bunsen burner until steam arose from the stain on the slide. The slides were steamed for about 3 minutes, replacing the malachite green as it evaporated from the slides, and were then allowed to cool for about 5 minutes before rinsing with water. Then the counter stain safranin-O was added and allowed to stand for one minute. The slides were then washed and allowed to air dry, and observed under oil immersion objective lens of a microscope. Spores appeared in green colour and the vegetative cells were pink in colour.

2.1.4.2.3 Mannitol Motility Test

Mannitol motility medium was prepared and about 3-4 ml was distributed in test tubes. The tubes were sterilized in an autoclave and left for setting in a vertical position. After cooling, the inoculum from the culture was
stabbed straight to the bottom. The tubes were then incubated at room temperature for 48-72 hours. Change of color from pink to yellow in the medium showed the utilization of mannitol. Motile bacteria moved away from the line of inoculation and exhibited diffused growth.

2.1.4.2.4 Marine Oxidation Fermentation [MOF] Test

MOF medium was prepared and about 5 ml was distributed in test tubes and sterilized in an autoclave. After sterilization, the tubes were kept in a slanting position. The inoculum was stabbed and streaked on the agar slant and incubated for 48 hours. Oxidative forms showed a change in colour from pink to yellow from slope to bottom. Gas production was observed by the presence of cracks and bubbles in the hard agar of the butt area. Alkaline reactions were noticed by a deep pink color in the slope region.

2.1.4.2.5 Catalase Test

On a clean glass slide, a smear of fresh bacterial culture was prepared. A drop of concentrated hydrogen peroxide (H₂O₂) solution was added on the smear. Effervescence or bubbling was noticed in the case of cultures producing catalase enzyme.

2.1.4.2.6 Oxidase Test

Small pieces of filter paper (Whatman paper No.1) were soaked in 1% aqueous solution of N,N,N’,N’-tetramethyl-p-phenylene diamine dihydrochloride and the papers were dried. A small portion of the culture was placed on the test paper with a clean platinum loop. Oxidase positive cultures showed a blue color within 10-30 seconds.
2.1.4.2.7 O/129 Test

This test was used to distinguish between *Vibrio* sp. and *Aeromonas* sp. Discs were prepared using 2, 4-diamino-6, 7-di-isopropylpteridine phosphate. The discs were placed on swabbed nutrient agar plates and incubated overnight at 30°C. Sensitive cultures showed a zone of inhibition around the disc.

2.1.4.2.8 Antibiotic (Kirby-Bauer) Test

Penicillin G sensitivity was tested to distinguish between *Pseudomonas* sp. and *Moraxella* sp. Muller Hinton agar plates were swabbed with 24 hour old bacterial broth and the antibiotic discs were placed and the plates were incubated overnight at 37°C. Clearing zone around the disc indicated sensitivity to the antibiotic.

2.1.4.3 Screening for hydrolytic enzyme production of bacteria

For the purpose of hydrolytic enzyme screening, the cultures were spot inoculated on nutrient agar medium supplemented with specific substrates viz. starch, tributyrin, gelatin, carboxy methyl cellulose, tannic acid, phenolphthalein diphosphate and sodium alginate and examined for enzyme production.

2.1.4.3.1 Amylase

The plate assay for amylase production was carried out using the method of Mac Faddin (1980). The bacterial strains were spot inoculated on to nutrient agar medium supplemented with 0.5% soluble starch and were incubated till the colonies were clearly visible. The plates were flooded with Gram’s iodine solution. The amylase producing colonies exhibited a clear zone against a blue-black surrounding.
2.1.4.3.2 Lipase

The plate assay for lipase production was carried out using the method of Sierra (1957). The strains were spot inoculated on to nutrient agar medium supplemented with 1% tributyrin and were incubated for 48 hours. Clearing zone around the colonies indicated a positive reaction.

2.1.4.3.3 Protease

Protease production was studied by using gelatin as the substrate (Smibert and Kreig, 1994). Nutrient agar medium supplemented with 2% gelatin powder was inoculated and incubated for 48 hours. The proteolytic activity was observed as a clearing zone around the colonies when flooded with 1% mercuric chloride solution.

2.1.4.3.4 Cellulase

Cellulase production was screened according to the method of Hankin and Anagnostakis (1977). The strains were spotted on to nutrient agar medium supplemented with 0.5% carboxy methyl cellulose. After incubation for 3-4 days the plates were flooded with congo red dye (1 mg/ml) solution. It was further incubated for a period of 15 minutes at room temperature. The plates were washed several times using 1 M sodium chloride to remove the unbound excess dye. A clearance zone against a bright red background indicated the production of cellulase.

2.1.4.3.5 Ligninase

The bacterial strains were spot inoculated on to nutrient agar medium supplemented with 0.5% tannic acid. After 4-7 days incubation the appearance
of a clearance zone around the colony was taken as an indication of ligninase production.

2.1.4.3.6 Phosphatase

The screening for phosphatase production was carried out by the method of Baird - Parker (1966). Basal nutrient agar plates containing 1 ml of 1% solution of phenolphthalein diphosphate were spot inoculated with the bacterial culture and incubated till sufficient growth was observed. These plates were then exposed to ammonia (NH₃) vapours by inverting it over a petridish containing NH₃ solution. Pink colouration of cultures indicated the presence of phosphatase enzyme.

2.1.4.3.7 Alginase

The screening for alginase production was carried out by the method of Gacesa and Wusteman (1990). Nutrient agar medium supplemented with 1.5% sodium alginate were spot inoculated with bacterial strains. After incubation for 2-3 days the plates were flooded with 10% cetyl pyridinium chloride solution. The positive strains showed a clearance zone against an opaque white background after incubation for 10-30 minutes at room temperature.

2.2 Statistical Analysis

Statistical interpretation of data was carried out by using softwares like MS Excel, PRIMER 6, SPSS 13 and Origin Pro 7.0.