

Chapter 3

MATERIALS AND METHODS

3.1 Study Area

3.1.1 Location and Description

Dona Paula is a semi-enclosed bay located on the eastern side of the Zuari estuary on the west coast of India. This small bay opens into the Arabian Sea and is sheltered on both sides by rocky cliffs. The bay and the adjacent mesotidal estuary are under the influence of the atmospheric monsoon circulation and experience a spell of heavy precipitation from June to September (south west monsoon) with a total seasonal rainfall of ~2500 mm. The tides are semidiurnal and the tidal height during spring tide is ~2.3m (Shetye et al. 1995; Sundar and Shetye, 2005). The average neap tide is of range ~0.7m. The wave height in this area is > 1.5 m during June – August and <0.7m during October – April (Chandramohan et al. 1997). This study deals with the diurnal and annual variation of DMSP concentrations in Dona Paula bay vis-à-vis the abiotic and biotic factors responsible for these variations. The objective is to understand the link between DMSP concentration and environmental variables (biotic and abiotic).

Dona Paula bay ($15^{\circ} 27' N$, $73^{\circ} 48' E$) is located at the terminus of Zuari estuary. It is a semi – enclosed bay and is not influenced by the seaport and riverine traffic. The depth of the study area varied from 3 to 5m (Fig. 1). It is prominently affected by the south west monsoon every year (Suprit and Shankar, 2008). The moisture laden south

westerly winds bring heavy rainfall. The hydrographic parameters of the bay differ markedly between the heavy run-off period of June - September and the low run-off season of November - May. The water column remains well mixed for most part of the year except during the southwest monsoon (SW Monsoon) season, when the surface layer becomes less saline (Qasim and Sen Gupta, 1981).

3.1.2 Sampling Strategies

Water samples were collected in polyethylene aspirator (5 l) during day time (between 06.00 to 15.00 hr) from April 2004 to April 2005. The sampling time was based on the Indian Standard Time which is ahead of the Greenwich Mean Time by 330 minutes. Sampling was done during the receding high tide to minimize tidal effects. The monthly tidal variation of Dona Paula bay is shown in Figure 2. The diurnal sampling was fixed for 16-17th December 2004 after careful monitoring of physico-chemical and biological parameters inclusive of tides. The weekly tidal variation of Dona Paula bay is shown in Figure 3. The monthly sampling dates were fixed after taking the tides into consideration. Prior to sampling, the aspirator were thoroughly rinsed with the surface water and the mouth of the aspirator was covered with 200 μm mesh in order to exclude mesozooplankton grazers and the tap was left open to release air during sampling.

3.2 Physical Parameters

3.2.1. Depth

The depth of the water column was measured with a graduated rope suspended with deadweight.

3.2.2. Temperature

Water temperature was measured with a field thermometer. The tidal data were measured using NIO tidal gauge. Alternatively tide at Dona Paula was also predicted using TASK 2000.

3.2.3. Salinity

Salinity was measured using a hand refractometer (ATAGO 2442-W01) calibrated to zero with distilled water or was measured with a Guildline 8400 Autosal salinometer. pH was measured using a digital pH meter (Thermoelectron corporation Orion 3 star) after calibrating it with the standard buffers of pH 4, 7, and 9.2 (Himedia) respectively.

3.2.4. Dissolved oxygen

The dissolved oxygen (DO) concentration in the water samples was estimated using Winkler's titrimetric method (Carpenter 1965). Water samples were collected in 125 ml acid washed (10% HCl) glass-stoppered bottles and fixed immediately on board with Winkler's

reagents (1 ml of manganous chloride (3M) and 1 ml of alkaline iodide (8M-4M) solution). While analyzing at the laboratory, 1 ml of sulphuric acid (10N) was added to the sample and was titrated with 0.01N sodium thiosulphate using starch as indicator. The procedure was standardised by using potassium dichromate. Results are expressed as ml l^{-1} .

3.3 Chemical Parameters

3.3.1. Nitrite

The amount of nitrite in the water sample was estimated following the procedure of Parsons et al. (1984). The nitrite was allowed to react with sulphanilamide in an acid solution. The resulting diazo compound reacted with N-(1-naphthyl)-ethylenediamine and forms a highly coloured azo dye. The extinction was read in a spectrophotometer at 543 nm. Standards were run with analytical reagent quality sodium nitrite. Results are expressed in μM .

3.3.2. Nitrate

Nitrate was estimated spectrophotometrically (Parsons et al. 1984). The water sample was passed through cadmium-copper column, where the nitrate chemically gets reduced to nitrite. The nitrite estimation was carried out as mentioned above. Potassium nitrate was used for standardization.

3.3.3. Phosphate

Inorganic phosphate was estimated using the method of Murphy and Riley (1962). Phosphate and ammonium molybdate are allowed to react in acid solution to give phosphomolybdic acid, which is reduced by ascorbic acid. Optical density was measured using a spectrophotometer after 10 min at 882 nm using a 10cm quartz cell. Potassium dihydrogen orthophosphate was used as for standard. The values were expressed in μM .

3.3.4. Total organic carbon (TOC)

For total organic carbon (TOC) analysis, 30 ml of water samples were transferred to clean polycarbonate bottles and immediately acidified with 1 ml of 80% ortho-phosphoric acid (pH=2) and stored in polycarbonate bottles at 4 °C. TOC content in the water samples was determined with a commercial Shimadzu TOC-5000 organic carbon analyzer. The system blank and instrument blank were determined using UV oxidised milli-Q water. Before injection, samples were acidified with HCl to pH 2 and sparged with synthetic grade air (≤ 0.1 ppm CO_2 , CO and hydrocarbons) (Speciality gases, Mumbai) to remove inorganic carbon. The water sample was injected into a platinum catalyst at high temperature (680–700°C) and the TOC was oxidized to CO_2 and measured in a non-dispersive infrared detector (NDIR) and computed for TOC concentrations following the

manufacturer's guidelines as well as Hansell and Peltzer, (1998). The instrument is equipped with high sensitivity Pt catalyst (0.5% Pt on alumina) and measured at 680°C. Triplicate injections of 100 µl were averaged for every sample (CV%=<2).

3.3.5. Organic Sulfur Compounds

Dimethyl Sulphoniopropionate and Dimethyl sulphide

Triplicate sub - samples for total DMSP were collected carefully in acid washed clean glass stopper bottles with no head space. The samples were held in dark at 4°C and were analyzed within 1 - 2 hr. DMSP was measured after purging for DMS. The degassed samples were kept for hydrolyzing for 6 hr using 10 M NaOH (pH after alkali addition was >12) (Turner et al. 1990; Shenoy, 2002). The released DMS was passed through moisture traps (ice bath, glass wool and potassium carbonate) and then cryogenically (liquid nitrogen) trapped in Teflon column. The column was then transferred to a water bath, maintained at >80°C, for elution of the trapped gases. The stripped DMS was estimated using a Hewlett Packard 5890 series II plus gas chromatograph equipped with a Chromosil 330 column (2.44 m; inner diameter 2.1 mm) and with a flame photometric detector (FPD). The oven and detector temperatures were 40 and 150°C, respectively. Repeat analysis of the same sample showed no detectable DMS indicating the complete hydrolysis of DMSP. All samples and

standards were analysed in triplicate with an analytical precision of 5% at 0.1nM of DMSP. DMSP was obtained from Research Plus, inc and standards were prepared in milli Q distilled water and analysed as described above. Tests revealed that about 95% of $DMSP_t$ was converted to DMS during the 6 hr alkali hydrolysis. Reproducibility of DMS and DMSP in seawater samples was periodically checked.

DMSP was also determined as acrylate after conversion to dimethylsulfide and acrylate by overnight treatment with 1 M NaOH (White, 1982) using C18 column (Econosil C18-5 μ , 250x4.6mm, Alltech) with 7.5 % acetonitrile in 1% (w/v) phosphoric acid as the mobile phase at a flow rate of 1ml/min in the pump Spectra system P4000(HPLC – Thermo Separation Products , SCM1000). The eluate was monitored at 205 nm (Jansen and Hansen, 1998) in Spectra system UV2000.

DMS Flux

The sea-to-air flux of DMS is generally expressed according to Liss and Merlivat (1986):

$$\text{Flux}_{(DMS)} = K_w \Delta DMS$$

where K_w is piston velocity or gas exchange coefficient ($cm\ h^{-1}$) and ΔDMS is the DMS concentration difference across the air-sea interface.

$$\Delta \text{DMS} = \text{DMS}_{(\text{air})} H^{-1} - \text{DMS}_{(\text{water})}$$

where, $\text{DMS}_{(\text{air})}$ is the concentration of DMS in air, which can usually be ignored because it is very low. $\text{DMS}_{(\text{water})}$ is the concentration of DMS in water and H is the Henry Constant, which is the ratio of the concentration of gas in air to its concentration in water at equilibrium.

$$\text{Hence, Flux}_{(\text{DMS})} = K_w \text{DMS}_{(\text{water})}$$

Nightingale et al. (2000) gave a formula for calculating K_w

$$K_w = 0.222u^2 + 0.333u (\text{Sc}/600)^{-0.5},$$

where u is daily averaged wind speed at 10 m height (m s^{-1}) and Sc is the Schmidt number, which is used to adjust piston velocity to a given gas and water temperature. The Schmidt Number for DMS in seawater was calculated by the equation given by Saltzman et al. (1993), where T is the temperature in units of degrees Celsius:

$$\text{Sc} = 2674.0 - 147.12 (T) + 3.726 (T)^2 - 0.038 (T)^3$$

3.4 Biological Parameters

3.4.1. Chlorophyll *a* and phaeopigments

Chlorophyll *a* was estimated based on the fluorimetry method as suggested by Yentsch and Menzel (1963). A known amount of water sample (250 ml) containing chlorophyll pigments was filtered in

diffused light condition through glass fibre filter (Whatman GF/F). Pigments concentrated on the filter was extracted for ≥ 24 h in ice cold 90% (v/v) acetone and was determined fluorometrically using Turner Designs fluorometer 10-000R at an excitation wavelength of 430 nm and an emission wavelength of 670 nm. Standardization was carried out by using chlorophyll *a* standards (Sigma, USA). For estimation of phaeopigments, the above sample was acidified with two drops of 1.2 M HCl and determined fluorometrically. Both chlorophyll *a* and phaeopigments values are expressed as $\mu\text{g l}^{-1}$.

Calculations for chlorophyll estimation

$$\text{Chl } a (\mu\text{g/ml}) = (11.6E_{665} - 1.31E_{645} - 0.14E_{630})/V$$

Where V= volume of sample filtered

3.4.2. Phytoplankton

Fixation and preservation:

Water samples (250 ml) were fixed with Lugol's solution (Hasle, 1978). Lugol's iodine is added in a ratio of 1 part to 100 parts of the seawater sample. Prior to microscopic analysis, samples were concentrated to 5–10 ml by carefully siphoning the top layer with a tube covered by a 10 μm Nitex filter on one end. Phytoplankton cell density was estimated by using Sedgwick Rafter Cell. The counting cell was filled

with the phytoplankton sample and placed on the mechanical stage of the microscope. Then the counting cell was left for about half an hour for proper sedimentation. The organisms were counted from one corner of the counting cell to other. Replicates of the samples were counted and average values were taken into account for the calculation. The total number of phytoplankton present in a liter of water sample was calculated using the formula

$$N = \frac{n \times v}{V} \times 1000$$

Where, N is the total number of phytoplankton cells per liter of water filtered, n = average number of phytoplankton cells in 1 ml of plankton samples, v = volume of plankton concentrate (ml), V = Volume of total water filtered.

3.4.3. Primary Productivity

Primary production was measured by ^{14}C assimilation method (Lohrenz et al. 1992). Three light and one dark (125 ml) acid washed bottles were filled with water samples. After inoculation with labeled $\text{NaH}^{14}\text{CO}_3$ (Activity: $5 \mu\text{Ci ml}^{-1}$, BARC, Mumbai) samples were incubated for 12 hrs from (6 am to 6 pm) at the sampling site. Following retrieval, the water samples were filtered immediately through $0.45 \mu\text{m}$ filter (Millipore, GS type) under diffused light and low

pumping pressure (<100 mm Hg). Radiolabeled dissolved inorganic carbon (DI^{14}C) was removed by exposing the filter to fumes of concentrated HCl for a minute. The filters were then placed in scintillation vials and 5 ml of scintillation cocktail in dioxane (Spectrochem, Mumbai) were added. Radioactivity was measured in a liquid scintillation counter (LKB Wallac 1209). The dissolved inorganic carbon (DIC) concentration ranged from 75 to 95 $\text{mg CO}_2 \text{ l}^{-1}$ during the non-monsoon and 15-20 $\text{mg CO}_2 \text{ l}^{-1}$ during the monsoon seasons. The above values were used for the calculation of primary productivity and the results are expressed as $\mu\text{g C l}^{-1} \text{ h}^{-1}$.

3.5. Bacteriological parameters

3.5.1. Total Counts (TC)

Samples for total bacterial counts were fixed immediately with buffered formalin on board and stored at 4 °C upon arrival to the laboratory. Bacterial abundance was determined by acridine orange direct count method (Hobbie et al. 1977). Water samples (2 ml) preserved with 2% (final concentration) buffered formalin were stained with acridine orange (Hi-Media, Mumbai) (final concentration 0.01% w/v) for five minutes before filtering it through 0.22 μm black stained Nucleopore filter (Whatman Asia Pacific, Singapore). Samples were enumerated at 1250x magnification in an Olympus (BH) epifluorescence microscope,

using a 515 nm barrier filter and at least 10 fields of >30 bacteria field⁻¹ were counted. Bacterial abundance is expressed as numbers per litre.

3.5.2. Total Viable Counts (TVC)

TVC were made following the method of Kogure et al. (1984). Water samples were incubated at 20°C for 8 hrs with nalidixic acid (0.02% w/v), piromidic acid (0.001% w/v), pipemidic acid (0.01%w/v) (Sigma, USA) and yeast extract (0.01%). Incubation was terminated by the addition of buffered formalin (final concentration 2%). Samples were stained with acridine orange (as mentioned for TC) and observed under epifluorescence microscope. Only swollen and elongated cells were enumerated as viable bacterial cells and their counts are expressed as numbers per litre.

3.5.3. Colony forming units (CFU)

For the enumeration of colony forming units (CFU), water samples were serially diluted in autoclaved 50% seawater blanks. 100 µl (10 or 100 fold dilution) of the sample was surface plated on Nutrient Agar medium (NA) (Hi-Media, Mumbai), prepared with 50% seawater. The plates were incubated for 36-48 hrs at room temperature (28 ± 2°C). Bacterial colonies were enumerated and expressed as colony forming units (CFU) l⁻¹.

3.5.4. Dimethylsulphoniopropionate (DMSPU) and Dimethyl sulphide Utilizers (DMSU)

Two bacteria populations, 'DMS' and 'DMSP' utilizers were enumerated by the Most Probable Number (MPN) method (Visscher et al.1991) with a slight modification of Visscher et al., (1991) medium by adding 0.02% of 2,3,5- triphenyltetrazolium chloride (Lenhard,1956). Visscher's medium consisted of the following (in grams per liter): NaCl (25.0), NH₄Cl (0.2), CaCl₂- 2H₂O (0.225), KCl (0.2), MgCl₂- 6H₂O (0.2), anhydrous KH₂PO₄ (0.02), and anhydrous Na₂CO₃ (2.0), supplemented with vitamin B₁₂ (20 pg liter⁻¹), FeSO₄ 7H₂O (1 mg liter⁻¹), trace element solution (1 ml liter⁻¹) of Widdel and Pfennig (1981) and 1mM of DMSP. The pH of the medium was adjusted to 7.5. After 3 weeks of incubation in dark, growth was determined in three replicates and scored by the method of de Man (1975). The numbers are expressed per litre. All tubes were also examined microscopically. The positive tubes were further transferred into fresh mineral medium plates and tubes containing 1 mM of DMSP as the only substrate to confirm utilization of DMSP (i.e. growth).

3.6. Identification

3.6.1. Phytoplankton

Generic and species identification of phytoplankton was done by their

gross morphology, special structures and shape according to the keys provided by Subramanyan (1946, 1968), Subramanyan and Sarma (1961), Lebour (1978), Desikachary and Ranjithadevi (1986), Desikachary and Prema (1987), Desikachary et al. (1987), Tomas, (1997).

3.6.2. Bacteria

Biochemical Characteristics

DMSP utilizers were only isolated at random as DMS utilizers were very fastidious and very slow growers. They were transferred to NA plates and checked for purity on the basis of microscopic examination. The isolates were stored in NA slant tubes at 4 °C for taxonomic identification and characterization. The physiological and biochemical properties were examined according to standard methods of Gerhardt et al. (1981). General cell morphology was studied under an Olympus inverted microscope using 3-day-old cultures of the strains grown on mineral media plates. The bacterial isolates were identified up to generic level following the scheme of Oliver, (1982).

3.7. Studies on five DMSP Utilizers.

3.7.1. Characteristics of five strains

Scanning Electron Microscope (SEM)

Five isolates were selected for detailed study based on cluster analysis and their ability to utilize DMSP. Suspensions of colonies cultured on 1 mM of DMSP were prepared in 0.9% sterile saline. Clean and sterile glass pieces were immersed in the suspensions and left overnight for the cells to form a biofilm on the glass surface. The glass pieces were retrieved and the biofilm was then subjected to dehydration by running it through a series of increasing acetone concentrations (10, 30, 50, 70, 90 and 100%). The samples were then air dried, mounted on a stub and sputter coated with Au/Pd. The specimens were seen using JEOL JSM – 5800 Scanning Electron Microscope. SEM was carried out only for 5 strains which were studied in detail.

Fatty Acid Profile

These were identified by phospholipid fatty acid analysis using the MIDI microbial identification system (MIS, NY). The strains for identification were streaked in Tryptic soy broth agar (TSBA). The cultured cells were gently scraped from the surface of the culture medium with a sterile 4 mm inoculating loop. The Saponification step involves the addition of a strong methanolic base (1.0 ± 0.1 ml of

Reagent 1) and heating. Thus, the fatty acids are cleaved from the cell lipids and are converted to their sodium salts. The methylation step involves the addition of methylation reagent (2.0 ± 0.1 ml of Reagent 2) which converts the fatty acids (as sodium salts) to fatty acid methyl esters. Fatty acid methyl esters are extracted using the extraction solvent (1.25 ± 0.1 ml of Reagent 3). A dilute base solution is added to remove free fatty acids and residual reagents from the organic extract. 2ul of sample is injected into the column (25 m silica capillary). The oven temperature is increased from 170C to 310 °C in each run. Since each fatty acid is sensitive to a particular temperature it gets volatilized at a particular retention time and gets detected by flame ionization detector (FID). The chromatogram is visualized with the CHEMSTATION software and transferred to software SHERLOCK for matching the fatty profile of the unknown organism.

16s rDNA Analyses

Total genomic DNA of these 5 bacteria was extracted by the phenol-chloroform method modified from Ausbel et al. (1987). The small-subunit 16 rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-ACGGCTACCTTGTTACGACTT-3'), which are complementary to the conserved regions at the 5'- and 3' ends of the 16S rRNA gene (Lane, 1991). The bacterial DNA (0.5 µg) was amplified by the PCR in a total volume of 50 µl

containing 0.5 U *Taq* DNA polymerase, 10 mM TAPS, pH 8.8, 3 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 10 pmol of each of the two primers and 200 μM each of dATP, dCTP, dGTP and TTP. The amplification of DNA was carried out in a hot-air rapid thermocycler (model no. 1833; Idaho Technologies) programmed for 40 cycles of denaturation at 94 °C for 10 s, annealing at 48 °C for 20 s and extension at 72 °C for 30 s. A final extension of 5 min was carried out at 72 °C. In all of the amplification reactions, water was used in place of DNA for negative controls. The amplified DNA fragment (1.5 kb) was separated on 1% agarose gel, eluted from the gel and purified using the PCR Purification Kit (QIAGEN). The purified PCR product was used directly for DNA sequencing.

Growth in different carbon compound

To test the ability to utilize 95 carbon sources of 150 μl of cell suspension of the five strains were inoculated into BIOLOG GN /GP plates (Ivanova et al. 1998). BIOLOG plates were incubated at 28°C. The results were read visually from day 1 to 5th day of incubation.as recommended by Ruger & Krambeck (1994). The results were recorded as + / -.

Growth at different temperature

The sample preparation of the 5 strains was done as mentioned earlier. The growth at different temperature (10, 18, 20, 22, 25, 37, 40 and 42°C) was determined in mineral medium containing 1mM of DMSP. The tubes were incubated for 5 days and growth was measured at OD_{600nm}.

Growth at different concentrations of NaCl

Sodium requirement was examined at 0, 3, 3.5, 10 and 15 % (w/v) NaCl. The period of incubation and growth measurements was done as mentioned above.

3.7.2. DMSP Utilization

Sample preparation

The bacterial isolates were inoculated in the nutrient broth and after 5 days of incubation were centrifuged at 10,000 rpm for 3 minutes. The cell pellet was washed with sterile normal saline, centrifuged again at 10,000 rpm for 3 minutes, was re-suspended in saline and the optical density was measured at 600 nm. An OD of 0.1 was equivalent to 15µg l⁻¹ of protein.

Growth on different DMSP related substrates

Fifty micro-litre (50 µl) of the above suspension was added to tubes

containing mineral medium amended with respective substrates at 10mM as shown in the table below. These substrates in the medium served as the sole carbon source for the bacteria. The substrates checked for growth studies were sucrose (6C), fatty acid (Butyric acid, 4C), amino acid (Cysteine, Sulfur containing compound; Succinate (4C), Methanol (1C) and organic compounds like Sodium pyruvate (3C), Sodium butyrate (4C), Lactic acid (3C), Sodium formate (1C), Citric acid (6C), Sodium acetate (2C). The other sulfur containing compounds used are Sodium thiosulfate, MPA, MMPA and DMSO.

Sl no	Substrate
1	Sucrose.
2	Sodium pyruvate.
3	L- Cysteine
4	Sodium butyrate
5	Lactic acid
6	Sodium formate
7	Succinate
8	Citric acid
9	Sodium acetate
10	Sodium thiosulfate
11	Butyric acid
12	Methanol
13	3-mercaptopropionate (MPA)
14	3- methiolpropionate (MMPA)
15	Dimethyl Sulfoxide (DMSO)

All the experiments were performed in duplicates. The tubes were kept on the shaker for eight days and OD was measured, (Shimadzu UV-1601, UV spectrophotometer). The isolates were subjected to statistical analysis (HCA & MDS) to group them on their affinity towards different substrates.

4. DMSP affinity at different concentrations and pH

The 5 strains were tested for its affinity towards DMSP at different pH and concentrations of DMSP using chemotaxis plate (Palleroni, 1976). The broth culture of bacterial isolates were grown overnight in 50% nutrient broth. The cells were harvested by centrifugation (Cooling centrifuge CRP 24) at 8000 rpm for 10 minutes at 17°C. The cell pellets were washed twice in saline and re-suspended in saline to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.6.

Chemotaxis

The concentration used were 300 & 60 µM ; 60 & 12 nM and 10 & 2 pM at pH 2, 6 and 7.5 in saline. Four chambers were excavated in a Lucite plate, measuring 5.5 cm per side and 1 cm thickness. Each one of the chamber consisted of two cylindrical compartments, measuring 7 mm in diameter and 5 mm in height, linked by a channel, 24 mm long , 2 mm wide and 2 mm deep. After proper cleaning, the plates were placed in a petridish after removing the lid and exposed to ultraviolet radiation for sterilization. The suspension of motile bacterial cells was

pipetted to fill the compartments and channel of each chamber. Each chamber was filled with 0.4 ml of the bacterial suspension. Bubbles in the chamber were avoided by filling the first compartment then sliding the tip of the pipette along edges of the channel while releasing the liquid, and finally filling the second compartment. Clean capillaries (1 μ l), which were sterilized by autoclaving, were kept in the petridish. Each capillary was picked up with forceps and filled with appropriate concentration of DMSP diluted in sterile milli Q distilled water. The capillary tubes were placed in such a way that it should be fully immersed in the suspension and its tips were centrally located in each of terminal compartments. A capillary filled with milli Q distilled water was included in each experiment as a negative control. The capillaries remained the chambers for 0.5 to 2 hrs in sterilized petridish. After the incubation period, each capillary was removed with thin forceps and washed externally with a thin jet of sterile distilled water. The washing was performed in the following way. The capillary was held with the tweezers in a vertical position, and the jet of sterile liquid is directed to it at an angle, pointing downwards, so as to wash the capillary from the middle of its length to its lower tip. The jet was interrupted, capillary was inverted, and washing was repeated on the second half of the capillary, again washing from the middle to the lower tip. After washing the contents, the capillaries were blown out into screw capped test tubes containing 10 ml of sterile distilled water. Fifty microlitre was

taken from each test tube and was spread plated on the 50% nutrient agar plates and kept for incubation for 48 hrs at room temperature (28 ± 2). The concentration of bacteria in each capillary tube was derived by counting the CFU on each plate and multiplying by the appropriate dilution factor.

DMSP utilisation and growth at varying pH, temperature and NaCl concentrations

The utilisation of DMSP by 5 strains was checked in varying pH (6.5, 7.5, 9), NaCl (1, 1.5, 2.0, 3.5, 4.0, 6.0) % (w/v) and temperature (10, 28, 37 °C). The bacterial suspension was inoculated into 2 ml headspace vials containing mineral medium amended with DMSP (500 µM). Zero hour reading was taken for DMSP and growth. All the experiments were conducted in triplicates. DMSP utilization was measured after conversion to acrylate by alkaline treatment (White, 1982). Acrylate was analyzed by HPLC after the cell suspensions were pelleted down by centrifugation. Growth was monitored by measuring cell protein. Appropriate controls were also kept.

Protein Estimation

Growth was determined by protein estimation of the culture using Folin – phenol reagent (Lowry's, 1951). The principle involves a two step reaction: Formation of copper-protein complex in alkaline solution (NaOH) which is then reduced to phosphomolybdate phosphotungstate

reagent to yield blue colour. The Folin's reagent is stable at acidic pH only. However the reduction indicated above occurs only at pH 10; therefore Folin's reagent is added to alkaline copper-protein solution. 1ml aliquot of the sample was taken in tubes. 5 ml reagent C was added and kept for 10 minutes. Folin's-phenol reagent was added and kept for 20 minutes in dark. The absorbance was determined at 750 nm (Jasco V-530 UV/VIS Spectrometer). Bovine serum albumin was used as standard (see Appendix).

Calculations

The amount of proteins per ml of the water sample was calculated by the given formula:

$$\text{Protein per ml} = \frac{(\text{Sample absorbance} - \text{blank}) \times \text{K factor}}{\text{Volume of aliquot taken}}$$

Where K factor = constant from standard graph.

Data Analysis

Spearman's correlation analyses were performed using the statistical package Stat Soft Inc. STATISTICA for Windows version 6 to find out the relationship between DMS (P), DMS (P) utilizers and various environmental variables. The multivariate analyses - PCA were performed using PRIMER version 6 (Clarke and Warwick, 1994). The variables which were observed throughout the study period only were considered. The variables were log (x+1) transformed before

performing PCA and only those variables with a load of > 0.1 were considered.

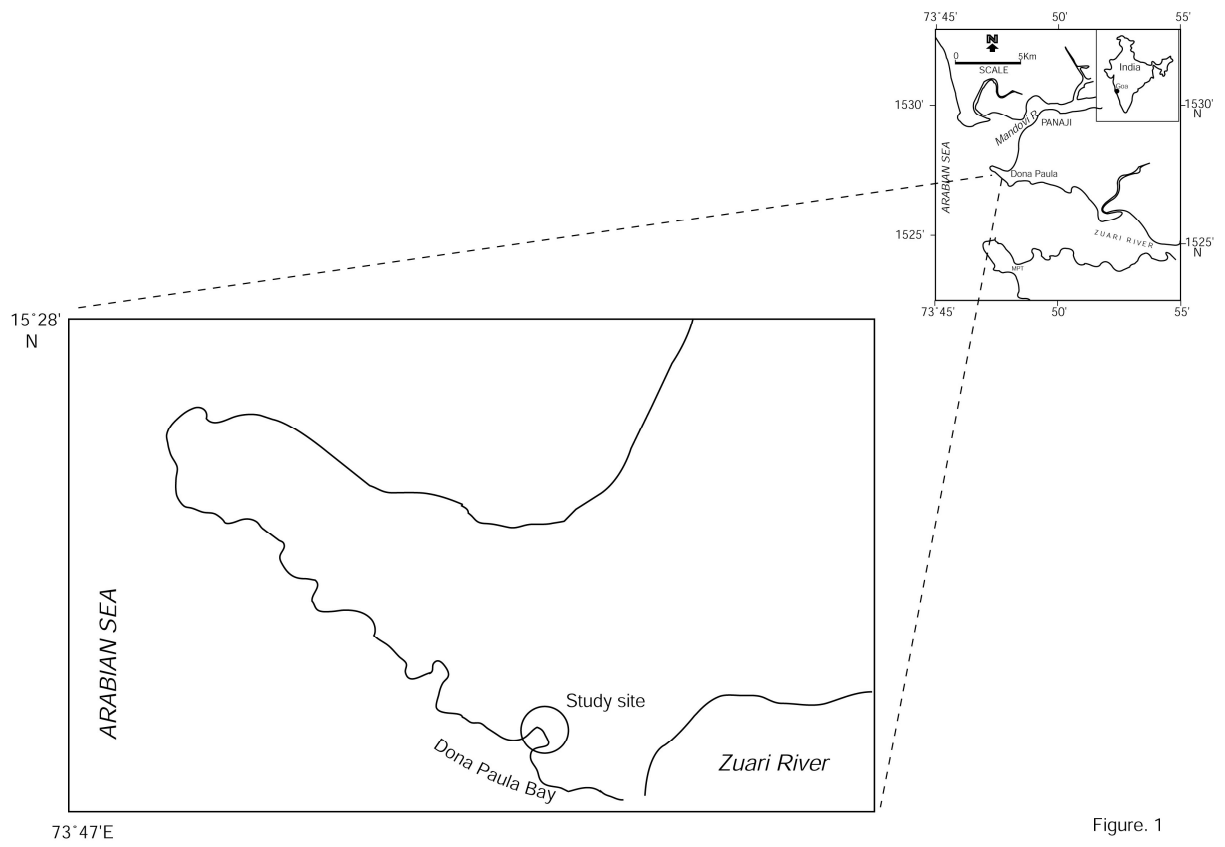


Figure. 1

Fig 1. Map showing the study site

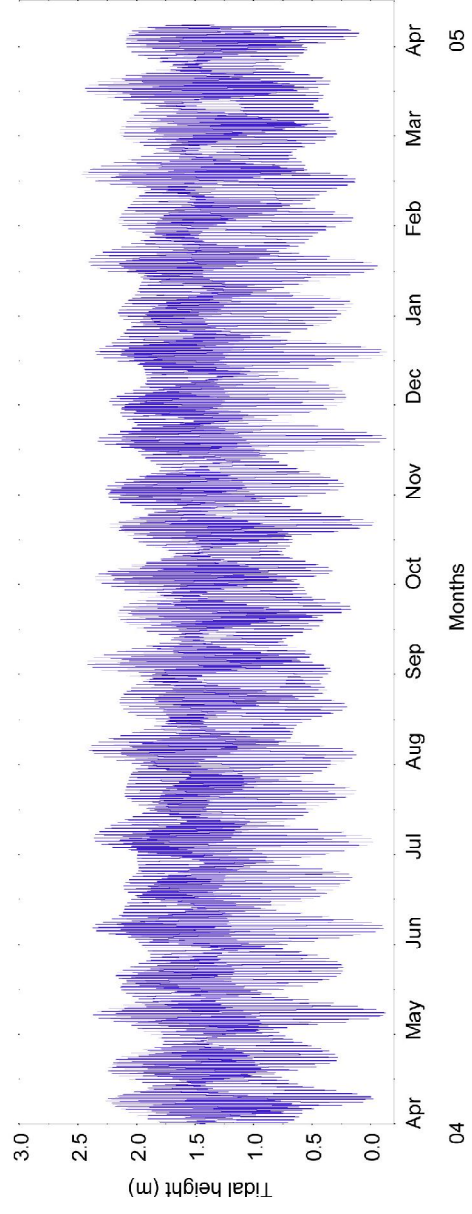


Fig. 2 Yearly tidal amplitude from April 2004 to April 2005 at the study site

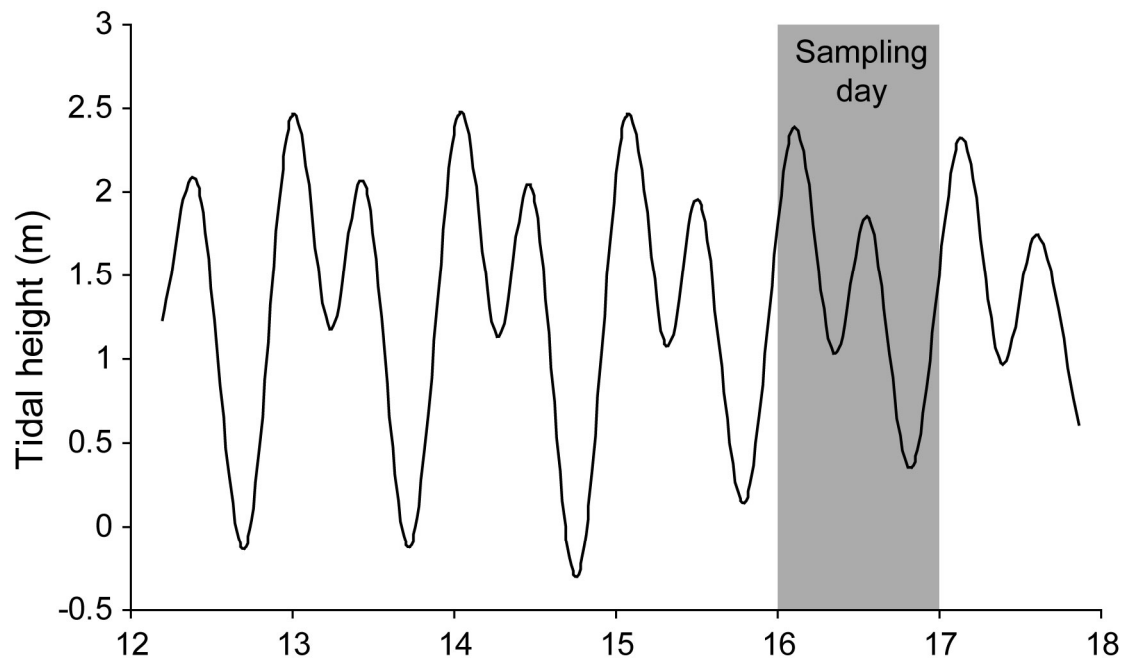


Fig. 3 Tidal amplitude from 12 to 18 December 2004