

CHAPTER 2

Materials and Methods

2.1. Study area

Goa, located along the central west coast of India, has a coastline of 151 km comprising 44% of sandy beach, 21% rocky coast, and 35% muddy flats (Kumar et al., 2006). Nine intertidal rivers traverse within the state of which two rivers Chapora in the North Goa and Sal in the South Goa are selected for the present study. Both Chapora and Sal estuaries are semi-diurnal micro-tidal estuaries with tidal amplitude of around 0.25 m during neap tide to 2.0 m during the spring tide (Fernandes et al., 2018).

The Chapora river, located in the Bardez taluka with a 32 km stretch of it influenced by salinity fluctuations and harbours around 220 hectares of mangrove forest (Goa State Pollution Control Board [GSPCB], 2019a). The estuary is surrounded by some popular tourist destinations like Vagator beach (South), Morjim beach (North), and Chapora fort (estuarine mouth). Additionally, manual sand extraction activity and aquaculture ponds are common in the estuarine regions. The Sal river in the Salcete taluka is 40 km long with a 14 km stretch of it under the influence of salinity harbouring 11 hectares of mangrove vegetation (Goa State Pollution Control Board [GSPCB], 2019b). The Sal estuary runs along the coastline, featuring shallow shoreline with a narrow mouth, which hinders the transport of domestic wastes into the sea (Fernandes et al., 2018). The Cutbona jetty, located around 2 km from the estuary mouth, is close to popular beaches like Cavellosim, Mobor and Betul, which run parallel to the estuary. Additionally, the collection of bivalves, mussels, and oysters by locals is a common practice along the estuarine banks.

Due to the inflow of population and tourism, anthropogenic activities such as fishing, river cruises, shoreline constructions, sewage disposal, sand mining, extraction of oysters, disposal of fish offal and polythene waste are increasing at an alarming rate. These activities are causing significant alterations to the habitats in these regions.

2.2. Sampling Sites

A total of four mangrove-associated intertidal stations, two each along Chapora (Station C1 and C2) and Sal (Station S1 and S2) estuaries were selected for the present study (Fig. 2.1. Table 2.1). Sites were selected based on habitat diversity, mainly variation in the mangrove vegetation and accessibility. Station C1 is located almost at the mouth of the Chapora estuary (1.5 km) and harbours young and scattered mangrove vegetation (Fig. 2.2). This station is indirectly affected by anthropogenic activities such as the deposition of litter along with the incoming tide and the influence of tourist boat rides. Station C2 is situated 4 km away from

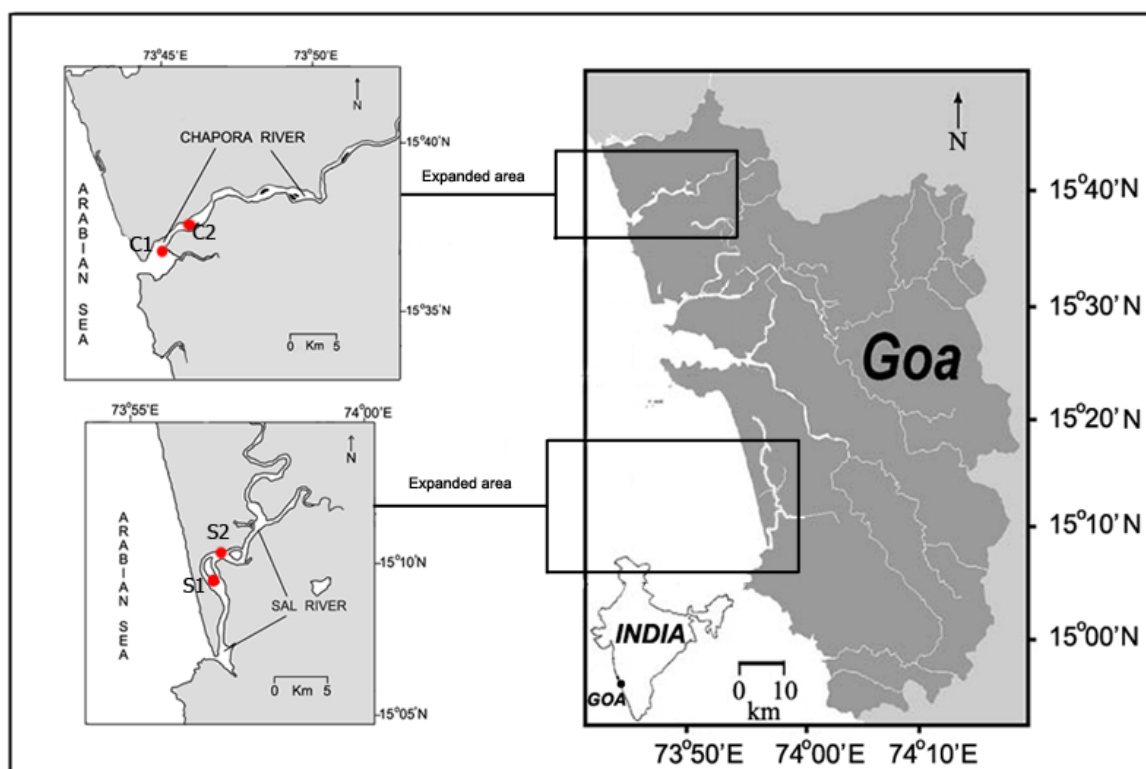


Figure 2.1. Map of the study area indicating the sampling locations.

Table 2.1. Details of sampling stations

Station no.	Name of Estuary	Geographical coordinates	Vegetation
C1	Chapora	15° 37.353'N, 73° 44.758'E	Sandy to muddy substratum with young, scattered <i>Avicennia marina</i> mangrove.
C2	Chapora	15° 37.953'N, 73° 45.765'E	Muddy substratum with dense, mixed mangroves (<i>Avicennia marina</i> , <i>Sonneratia alba</i> and <i>Rhizophora mucronata</i>) and associated shrubs (<i>Clerodendrum inerme</i> and <i>Acanthus ilicifolius</i>).
S1	Sal	15° 10.095'N, 73° 56.781'E	Muddy substratum with dense, monotypic mangrove <i>Avicennia marina</i> .
S2	Sal	15° 09.836'N, 73° 56.952'E	Sand flat lined by <i>Avicennia marina</i> mangroves.

the mouth of the estuary. This site is constantly affected by human activities such as the disposal of fish offal and polythene waste, and the movement of fishing canoes of the fisherman. Station S1 is located 3 km away from the mouth of the Sal estuary. The area around the station is surrounded by numerous hotels and resorts, along with shrimp farms. Station S2 is a sand flat located around 4.5 km away from the mouth of the estuary and is relatively pristine.

2.3 Sampling methodology

Intertidal surveys at each station were carried out during low tide at monthly intervals from April 2016 to March 2017 (Table 2.2). The sampling sites were accessed by a motorised canoe. Each survey comprised laying of 1 m² quadrant at low, mid and high tide levels to compute epifaunal density and record the types and numbers of brachyuran crabs and molluscs. Species abundance for burrowing fiddler and dotillid crabs was determined directly from the burrow counts; for sympatric crabs (different species occupying the same habitat) was determined with visual counts and photographs in addition to burrow counts and for sesarmids and running crabs with visual counts and photographs. Subsequently, representative subsamples of the biological specimens were picked and stored in pre-labelled containers.

The temperature of water and sediment was recorded on the field using a laboratory thermometer ($\pm 0.1^{\circ}\text{C}$ accuracy). Additionally, samples for estimation of salinity, pH, nutrient concentration, dissolved oxygen, chlorophyll-*a*, suspended particulate matter concentration in seawater and grain size, organic carbon, soil moisture, and chlorophyll-*a* concentration in sediment were collected. For fixing the dissolved oxygen in the water sample, 1 mL Winkler's reagent was added to 125 mL water sample in glass stoppered bottles. For assessing the chlorophyll-*a* pigment concentration, water and sediment sample was collected in amber-coloured polythene bottles. Water samples for recording other environmental parameters were collected separately in plastic bottles. Sediment samples were collected using scoops in pre-labelled polythene bags and stored in ice. Additional details of mangroves and associated vegetation and microhabitats were noted at every tidal level. Sample leaves and flowers were collected from the vegetation for further identification. All the samples were transported in an ice box to the Marine Biology Laboratory at Goa University for further analysis.

Table 2.2. Details of sampling carried out in the study area

Sr. No.	Month and Year of Sampling	Estuary	Date	Time of sampling (h)	Tidal amplitude (m)
1.	April 2016	Chapora	13/04/2016	10.00-12.00	0.3
		Sal	14/04/2016	10.00-12.00	0.4
2.	May 2016	Chapora	13/05/2016	09.30-11.45	0.5
		Sal	14/05/2016	11.00-13.00	0.6
3.	June 2016	Chapora	10/06/2016	8.30-10.45	0.3
		Sal	11/06/2016	9.00-11.00	0.5
4.	July 2016	Chapora	12/07/2016	9.45-12.00	0.7
		Sal	11/07/2016	9.15-11.15	0.8
5.	August 2016	Chapora	10/08/2016	9.15-12.15	0.8
		Sal	09/08/2016	8.15-10.15	0.6
6.	September 2016	Chapora	08/09/2016	9.00-11.00	0.8
		Sal	07/09/2016	9.00-11.00	0.7
7.	October 2016	Chapora	07/10/2016	9.00-11.00	0.8
		Sal	08/10/2016	8.30-10.30	0.8
8.	November 2016	Chapora	20/11/2016	9.30-11.30	0.7
		Sal	21/11/2016	8.30-10.30	0.7
9.	December 2016	Chapora	07/12/2016	9.00-11.00	0.7
		Sal	06/12/2016	9.00-11.00	0.7
10.	January 2017	Chapora	05/01/2017	9.00-11.00	0.5
		Sal	04/01/2017	8.30-10.30	0.6
11.	February 2017	Chapora	04/02/2017	9.45-11.45	0.4
		Sal	03/02/2017	9.00-11.00	0.4
12.	March 2017	Chapora	05/03/2017	9.00-11.00	0.3
		Sal	04/03/2017	9.00-11.00	0.2

2.4. Laboratory procedure

Taxonomic identification and biomass estimation

All the collected specimens were brought to the laboratory and washed under running tap water to remove debris. The larger specimens were photographed using a Sony RX 10II camera, and smaller specimens and body parts were photographed using a DIGITAL SIGHT DS-Fi2 camera attachment for the NIKON SMZ 745T stereomicroscope. Thereafter, standard morphometric characters (Carapace length and width for brachyuran crabs; shell length and width for molluscs) were measured using INSIZE digital vernier calliper, 0-150mm. Subsequently, the specimens were subjected to biomass measurement (wet weight) g per m² using electronic weighing balance SHIMADZU ATX124 for weight less than 10 gm and KERN-FCB for weight more than 10 gm weight of the specimens.

The species level identification was carried out using published identification keys, monographs and taxonomic literature. For brachyuran crabs identification, the literature referred included Alcock (1896, 1898, 1899, 1900, 1901); Kemp (1917, 1919); Chhapagar (1957a, 1957b); Banerjee (1960); Serène and Soh (1970); Wee and Ng (1995); Keenan et al. (1998); Ng, (2007); Barnes (2010); Lai et al. (2010, 2013); Padate et al. (2010); Sakai and Holthuis, (2013); Kaullysing et al. (2015); Ng et al. (2017); Shih et al. (2018); Innocenti et al. (2020).

Molluscs specimens were identified following literature such as Gruneberg (1978) Cernohorsky (1984), Reid (1986, 2001); Melvill (1893); Swennen (1997); Tan and Clements (2008); Jagtap et al. (2009); Huber (2010, 2015); Claremont et al. (2013); Ramakrishna and Dey (2010); Reid and Ozawa (2016); Arathi et al. (2018); Nerurkar et al. (2020); Goulding et al. (2021); Ravinesh et al. (2021); Tudu et al. (2021), Hussain et al. (2022).

The species names were updated from the online database World Register of Marine Species (WoRMS) (<http://www.marinespecies.org>). Further, photographic plates were prepared using Adobe Photoshop (version CC 2019). Abbreviations used in the present study include CW, carapace width; CL, carapace length; G1: male first gonopod for brachyuran crabs and SW, shell width; SL, shell length for molluscs.

Sample preservation

Brachyuran crabs were preserved in 5 % buffered formalin (buffered with hexamethylene tetramine) and molluscs were preserved in 5 % formalin. Samples were stored in plastic bottles and deposited as reference vouchers at the Marine Biology Laboratory, Marine Science programme at the School of Earth Ocean and Atmospheric Sciences, Goa University.

Analysis of physico-chemical parameters of water and sediment

1. Salinity (PSU)

The salinity of the water was measured by HANNA HI96822 Seawater Refractometer, calibrated to zero with distilled water with ± 0.1 PSU accuracy.

2. pH

A portable benchtop pH meter (Model: Thermo Orion Star A211) was used to measure the pH of water samples (Accuracy of ± 0.01). The pH meter was regularly standardised against standard buffer solutions (pH 4.0, 7.0 and 9.2).

3. Dissolved oxygen (mg/L)

The dissolved oxygen (D.O.) concentration of the water samples was measured following Winkler's titrimetric method (Grasshoff, 1983). The water samples fixed on field with Winkler's A and B reagents were treated with 50% HCl to dissolve the precipitate and titrated with standard sodium thiosulphate solution, where the endpoint (blue to colourless) was marked using starch as an indicator.

4. Suspended particulate matter (mg/L)

Suspended particulate matter (SPM) of water samples was measured by filtering 500 mL of sample through pre-weighed Millipore 0.45 μm membrane filter paper. The filter paper was then oven-dried at 100°C. Once dried, weight was recorded and subtracted from the initial weight to get the SPM value (Grasshoff, 1983). Filter papers were weighed using SHIMADZU ATX124 weighing balance with an accuracy of $\pm 0.001\text{mg}$.

5. Nutrients ($\mu\text{mol/L}$)

The essential dissolved nutrients (Nitrate-($\text{NO}_3\text{-N}$), Nitrite ($\text{NO}_2\text{-N}$), Phosphate ($\text{PO}_4\text{-P}$), and Silicate ($\text{SiO}_4\text{-Si}$)) were analysed in water samples following the procedure by Grasshoff et al. (1983). Calibrations were made using aqueous standards and results were expressed in

$\mu\text{mol/L}$. The procedure adopted for analysing each nutrient parameter is provided below briefly:

a) Nitrate and Nitrite

Nitrate and Nitrite in water were measured by colorimetric method, wherein Sulphanilamide (0.5 mL) and N (1-naphthyl)-ethylenediamine dihydrochloride (0.5 mL) were added to a 25 mL sample resulting in the formation of pink coloured azo dye. The optical density of the resultant sample was measured spectrophotometrically after 10 minutes at 543 nm in a 1 cm quartz cuvette. For nitrate, the sample (50 mL) was initially passed through the reductor Cadmium column (> 95 % efficiency) where the nitrate is reduced to nitrite.

b) Phosphate

Phosphate in water was estimated by acidifying 25 mL of sample with acid molybdate reagent (0.5 mL) and further addition of ascorbic acid (0.5 mL) giving blue coloured compound, which is measured spectrophotometrically at 880 nm using 1 cm quartz cuvette in 10 minutes.

c) Silicate

Estimation of silicate in water involved acidifying the sample (25 mL) with acid molybdate reagent (1 mL), thereafter treating it with ascorbic acid (0.5 mL) and oxalic acid (1 mL). In 20 minutes, the resultant blue silico-molybdate complex was measured at 810 nm with 1 cm quartz cuvette spectrophotometrically.

6. *Chlorophyll-a in water (mg/L) and sediment (mg/m³)*

Estimation of Chlorophyll-*a* in water and sediment was carried out following procedure by Strickland and Parsons (1972). 500 mL of water sample was filtered through 47 mm GF/F filter paper (Millipore, 0.7 μm pore size). The pigments were extracted by immersing the filter papers in 10 mL of 90 % acetone at 4 (± 2) °C for 18 h. For sediment samples 1 gm of sediment was weighed and was similarly immersed in 10 mL of 90 % acetone. Following the extraction, filter papers were centrifuged at 1500 rpm for 5 minutes. The clear supernatant liquid was decanted and measured spectrophotometrically at Wavelengths 665 nm, 645 nm and 630 nm.

7. *Texture of sediment: (Sand (%); Silt (%); Clay (%))*

The grain size distribution i.e. sand (> 63 microns), silt (2.0 to 63 microns) and clay (< 2.0 microns) fractions in sediment samples were estimated by standard pipette method (Folk, 1968). The abundance of each fraction was expressed in terms of percentage of total weight.

10 gm of oven-dried sediment was weighed and 1000 mL of distilled water was added and stirred thoroughly. Upon settlement of the sediment, the supernatant water was decanted and this step was repeated thrice. The fourth time, after decanting the supernatant water, 10 mL of 10 %, sodium hexametaphosphate was added and kept overnight with occasional stirring to break the flocculated clay particles. Further, 5 mL of 30 % Hydrogen peroxide (H₂O₂) was added to oxidize the organic matter. The resultant sample was then passed through a 63-micron sieve to separate the sand fraction. The filtrate after separating the sand fraction was collected in a 1000 mL cylinder, homogenized using a stirrer and left undisturbed to settle at room temperature. At 8 φ, 25 mL of filtrate was pipetted out at a depth of 10 cm from the 1000 mL mark following the standard table given by Folk (1968) (Kwankam et al., 2021). Collection for both sand and clay was done in pre-weighed beakers, which were dried at 60°C and weighed again to determine the sand, silt and clay fractions.

8. *Sediment Moisture (%)*

Sediment moisture was estimated using a thermogravimetric method as described by Topp et al. (2008). According to this method, a moist sediment sample is weighed and oven-dried for around 24 hours at 105 °C and reweighed. The weight loss is calculated to determine the moisture content.

9. *Total organic carbon (%)*

To estimate total organic carbon (%), sediment was dried and finely ground using mortar and pestle. 0.2 gm of the sample was weighed and 10 mL of chromic acid was added and kept in a boiling water bath for 15 minutes to oxidise all the organic carbon compounds present in the sample. Once the contents had cooled down, 200 mL of distilled water was added with 1 drop of Ferrous-phenanthroline indicator and titrated with 0.2 N Ferrous ammonium sulphate, until the pink colour persisted (El Wakeel & Riley, 1957) (Rehitha et al., 2017).

2.5. Data Analysis

Species abundance (numbers per m²) was computed from the burrows, visual counts and photographs of the quadrants. Subsequently, the abundance and biomass of the representative subsamples of each species were extrapolated to the total sample and used to draw inferences on the spatiotemporal variations. Monthly data collected was grouped into

seasons as Pre-monsoon (February-May), Monsoon (June-September) and Post-monsoon (October to January) following Sivadas et al. (2011).

Shannon-Weiner diversity index (H') was calculated using PAST version 4.13 statistical software (Hammer et al., 2001). H' measures the overall diversity or heterogeneity of species in a community, taking into account both species richness (the number of different species) and species evenness (relative abundance of each species) (Shannon & Wiener, 1963). Analysis of variance (ANOVA) (two-way, without replication) was carried out to assess the spatial and temporal differences in the diversity, abundance and biomass of brachyuran crabs and molluscs using the MS Excel program.

Redundancy Analysis (RDA) was performed to understand the spatial distribution of brachyuran crabs and molluscs and their relationship with environmental parameters. To know the suitability for the present study Detrended Correspondence Analysis (DCA) was carried out first and from the results obtained the choice of Canonical Correspondence Analysis (CCA) or RDA was made. The criteria for selection is based on the length of the first axis: if the length is > 2.0 CCA is performed, if it is < 2.0 RDA is performed (Pujari et al., 2021). For the present study length was < 2.0 , therefore RDA was performed. To perform the above analysis CANOCO 4.5 software was used.