CHAPTER 3: MATERIALS AND METHODS

3.1 STUDY AREAS

3.1.1 COASTLINE OF GUJARAT

Indian western state "Gujarat" faces the Arabian sea and the Arabian sea wash 1650 km of the state's water-giving it the longest coastline among all maritime Indian states and the country's longest coastline with a continental shelf area of approximately 1, 64,200 km^2 (35.3% of the country) and an Exclusive Economic Zone of 2, 14,000 km^2 (9.9% of the country). The coast of Gujarat consists of Deccan traps and tertiary rocks and in places of recent Pleistocene age alluvium with calcareous stone (Jhas *et al*., 2009). Gujarat coastline ranges in substratum characteristics such as sandy beaches, rocky shores, muddy flats and marshy areas with a percentage range of about 28%, 21%, 29% and 22% respectively (Misra and Kundu, 2005). Due to geographical location variations, parts of the Gujarat coastline show different properties not only in terms of geological characteristics, but also in terms of living biota. Because of its geography topography and hydrodynamic properties, Gujarat coast offers a broad range of opportunities for ecological and anthropogenic activities, promoting sustainable growth of a wide variety of marine water fauna and flora. The abiotic factors affecting the coastal environment vary widely. Gujarat coastline can mostly be divided into three major geographical sections, two main Gulfs Specially Gulf of Khambhat and Gulf of Kachchh, and the coastline of Saurashtra. For marine biologists, the Gulf of Kachchh has always been an important area of research as it shows the presence of live corals along with four other locations in India.

3.1.2 TOPOGRAPHY OF SAURASHTRA COASTAL AREA

The coast of Saurashtra has one of India's largest continental shelves with silent varied geographical and hydrological characteristics leading to different environmental variables in small spatial areas. The northern coast (Jamnagar-Okha) of Saurashtra trending E-W overlooks the Gulf of Kachchh having a

crenulated rocky shoreline with the sub-tidal zone comprises of channels, shoals, submerged islands, sand-bars, coral reefs and mangroves. The Saurashtra south-western coast (Jamnagar-Diu) faces the Arabian Sea. The coast has a generally straight NW-SE trend from Dwarka to Veraval, marked by well-developed sandy beaches. The characteristics of the shoreline between Veraval and Diu (E-W) are identical to those on the Dwarka arid coast of Veraval. The coastal stretch east of Diu to Bhavnagar reveals an open sea-to-gulf transition. Thus, Coast topography varies along the coast of Saurashtra, the northern coastline of the coast of Saurashtra is rocky and sandy, while the southern coastline is muddy with strong sediment load. The coastline of Saurashtra is the study area for the proposed intertidal rocky zone research. The main substratum component of Saurashtra coast is the mileolite lime stone and the existence of carbonates that leads to the development of organisms which create calcium carbonate exoskeletons (Lele, 1973; Merh and Chamyal, 1993; Bhatt, 2003 and Jha 2009). Many places are well known for fishing throughout the Saurashtra coastline, and there are also many harbors that provide the fishing community with livelihoods. The industrial complexes, however, still play a major role in deciding the form of community structure at certain locations. The coastal zone of Saurashtra coast in general and the rocky intertidal zone in particular is thus of much importance with a view of ecological assessment.

3.1.3 INTERTIDAL CHARACTERISTICS OF STUDY SITES

The locations selected for the proposed work are situated on the coastline of Saurashtra off the Arabian Sea, which are substantially rocky with irregular sand or mud patches (Fig. 3.1). Based on habitat presence of sea anemone fauna, a total 12 locations were studied with intertidal properties of the coast with special reference to sea anemone faunal diversity and distribution. Among these sites, Okha, Dwarka, Sutrapada and Vadodra jhala from north to south respectively were selected to investigate eco physiological changes within sea anemones.

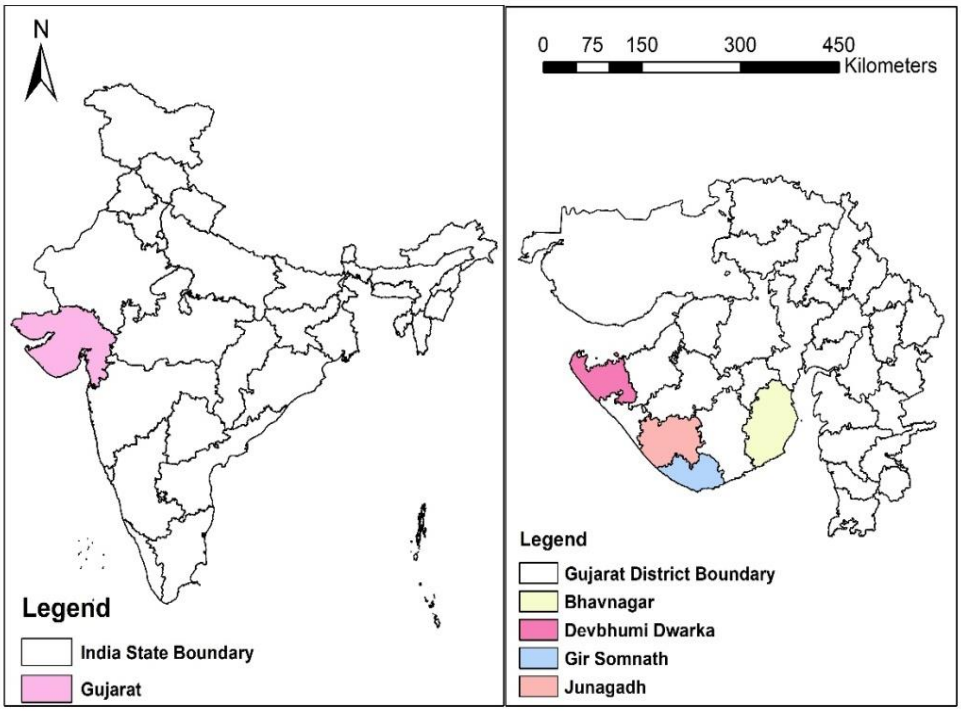

Figure 3. 1 : District wise study site locations along the Saurashtra coast

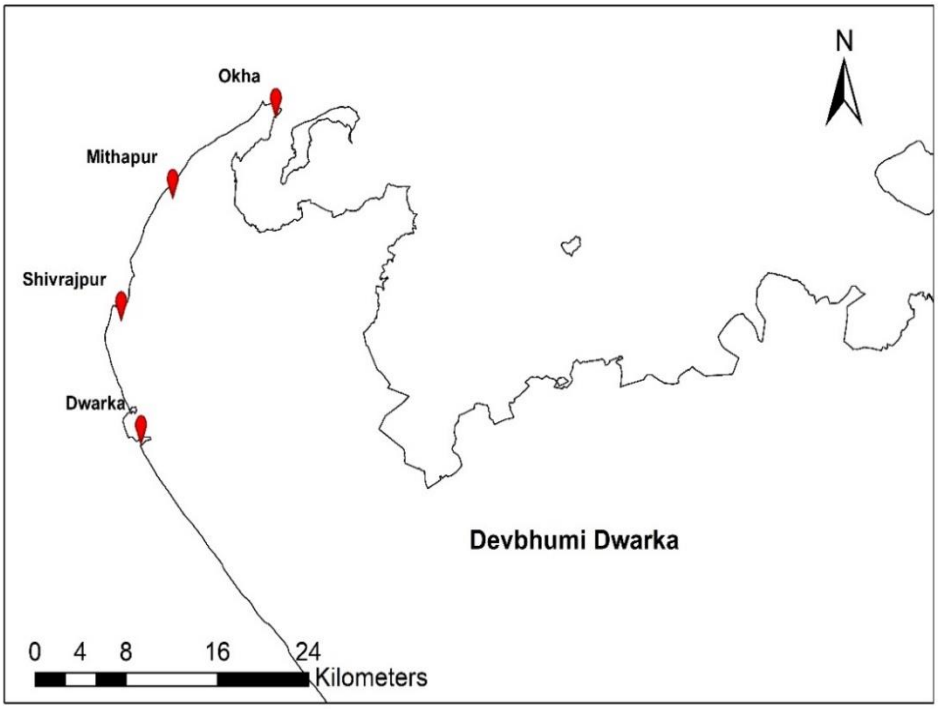

Figure 3. 2 : Location map for study sites of Devbhumi Dwarka District

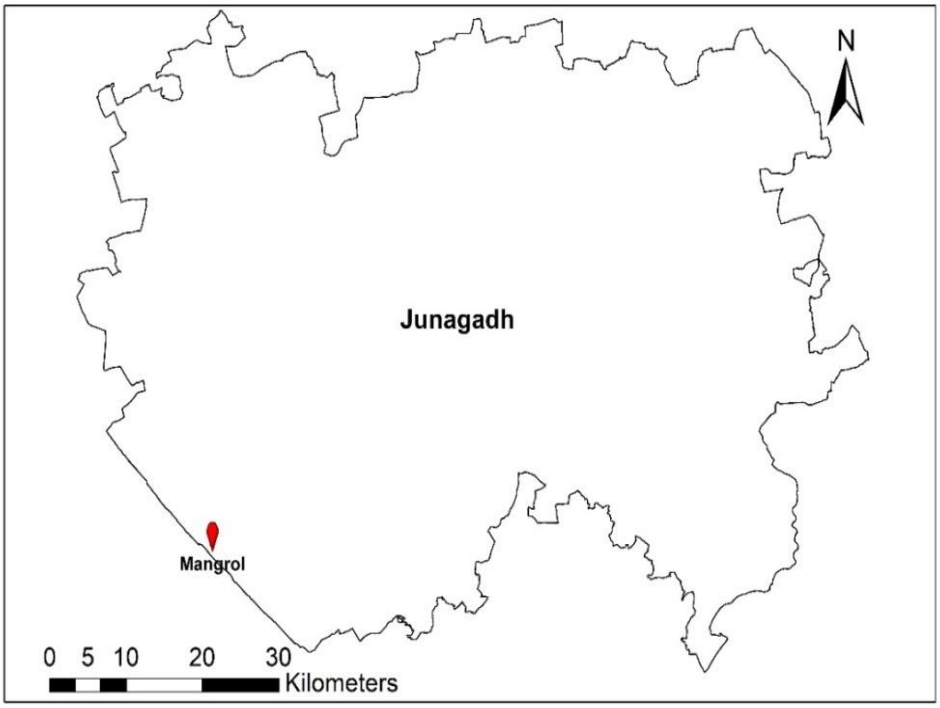

Figure 3. 3 : Location map for study site of Junagadh District

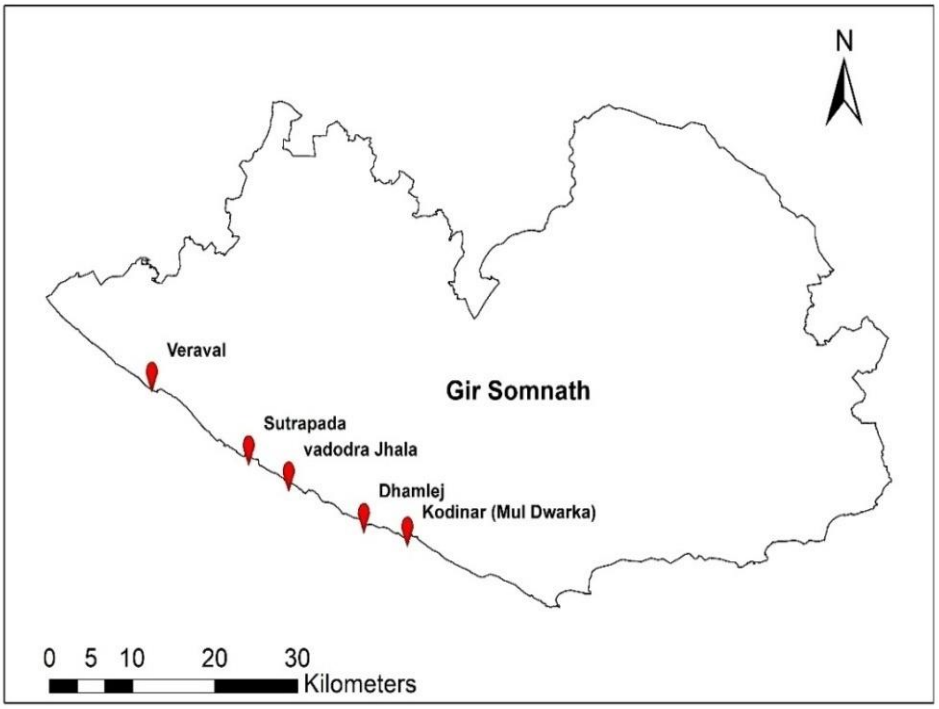

Figure 3. 4 : Location map for study sites of Gir Somnath District

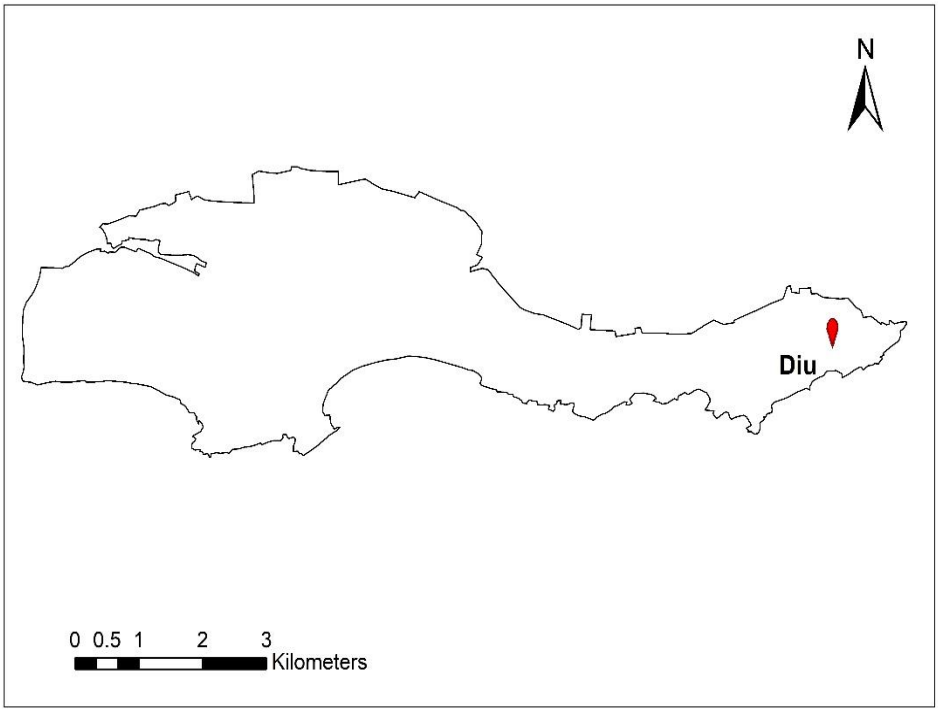

Figure 3. 5 : Location map for study site of Diu

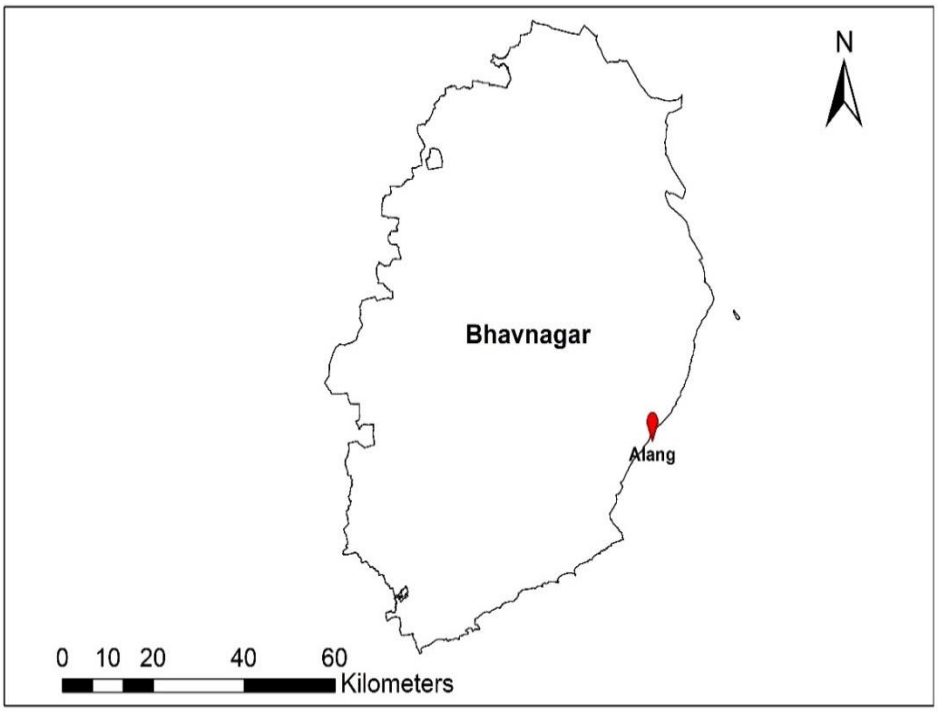

Figure 3. 6 : Location map of study site of Bhavnagar District

Site-1 Okha

Okha is situated at the outer rim of Gulf of Kachchh and forms the northernmost part of the Saurashtra coast. The sequence of alternating tides results in this habitat being inevitably submerged and emerging. The sandy beach gradually slopes into the rocky inter tidal area from landward side. The upper vertical zone is sandy-rocky whereas the middle zone is completely rocky and the lower intertidal zone comprises hard rocky substratum with sandy-rocky patches. The intertidal belt is interspersed with various microhabitats such as shallow tide pools, crevices, coral pools Zoanthid bed and algal bed. The shoreline represents the dissected reef limestone rock, which provide best suitable substratum for the growth of Seaweeds and marine benthic fauna. The broader intertidal zone of Okha experiences high sediment load, strong water currents and high turbidity due to Arabian Sea hydrodynamics as well as Indus delta inputs. The exposure area of the intertidal zone varies from 300 to 400 meters during the low tide (Fig. 3.2, Plate: 3.1 (1)).

Site-2 Mithapur

Mithapur is situated about 10 km from the Okha Port in the semiarid coastal belt of Saurashtra. As the Mithapur Bay is an integral part of the Gulf, the processes in the Gulf regulate its dynamics and ecology. Tides of Mithapur are mixed semi-diurnal with two unequal high waters and two unequal low waters that occur each day of the tide. The tidal water flows from the south-west into the Gulf and affects the north-west bay of Mithapur. This town is essentially a colony which is supported by Tata Chemicals Ltd. (TCL), one of the flagship companies of TATA Group. The natural sediment of intertidal area of the Mithapur has variable texture but usually consists of fine sand mixed with silt and clay. Highly heterogeneous, the sub tidal sediment ranges from coarse sand and calcareous debris to fine silt and clay. The information available on stratigraphy reveals that the strata below the thin surface layer of the sediment are generally made of calcareous clay. The coast has unique marine and coastal resources comprising rich coral reef, turtle nesting site, lush

mangrove cover and bird nesting site. The coast of Mithapur is above a 10 km^2 rocky land cover having rich diversity of coastal flora and fauna (Fig. 3.2, Plate: 3.1 (2)).

Site-3 Shivrajpur (Kachhigarh)

Shivrajpur (Kachhigarh) is located on the southern tip of the Marine National Park and Sanctuary and is about 21 km from Okha, facing the open Arabian Sea. The sea here is marked by high wave energy and longshore currents with good surfing activity. The characteristic feature of the area is shallow lagoon and water pool development during low tide. The landward side of the bays has a narrow but well-defined, crescent-shaped, sandy beaches with calcareous sand of medium to fine size. The central part of the lagoon has a sandy bottom, while the hard rock on the north and south of the bays has been partly exposed to land collapsing into rocky cliffs. Shallow lagoons with rocky bottom marked by several water pools provided an ideal habitat for various stony coral species in Kachhigarh as they protect corals from strong wave action (Fig. 3.2, Plate: 3.1 (3)).

Site-4 Dwarka

Dwarka is located about 30 km southwest of Okha, on the west coast of the Okhamandal Peninsula on the right bank of the Gomti River. Dwarka is one of the Hindus' most important religious centres and is considered to be one of India's four Dhamas on the west coast. This region is influenced by more tidal effects of the Arabian Sea due to its location near the mouth of GoK. The Arabian Sea tide has an effect on both the open and the turbulence of the gulf tide. Intertidal area of Dwarka is exposed to approximately 70-80 m during low tides (Light house area is selected as study site). The mineralogical and petro-graphic studies demonstrated the presence of calcareous sandstones that form the intertidal zone (Lele, 1973). For ages, there has been a steady deposition of sediments. Due to continuous water movements, creeks are also formed throughout the region. With wide and deep caves, the outer intertidal zone is highly elevated due to heavy wave action. The upper zone is covered

by the boundary of rock and sand, the middle zone with the coralline bed, the algal bed and the Zoanthid bed and the lower zone mostly submerged by seaweed. Intertidal belt is made up of large rocks and boulders that cause resistance to the upcoming wave and thus produce harsh splash of water (Fig.3.2, Plate: 3.1 (4)).

Site-5 Mangrol

Mangrol is located around 50 km west of Veraval. Mangrol is a tiny hamlet and a large fishing port situated along the coastline with many small-scale fishing industries. Fishermen's societies primarily use this side for exploitation during low tide for harvesting fish bait etc. In addition, it is also used as a dumping ground for fishing waste, broken nets, discarded plastics and boat parts etc. Mangrol has a completely rocky intermesh coast with a small mixture of sand, but without mud or silt, as an important coastal feature, there is a very straight edge between sand and rocky substratum. The upper part of the intertidal belt is usually covered with a silt and sand admixture combined with pieces of broken shells. The rocks are typically calcareous, famous milliolite rocks are the concentrated ancient equivalent of these biogenic sands. The milliolite shows the beach sands as cliffs; wave cut platforms and underwater dunes all along the shoreline suggesting quaternary variations in sea level (Stanley, 2004). Mangrol coast has a very long rocky intertidal zone which provides fine sheltering ecosystem to most of the molluscs and other benthic fauna (Fig. 3.3, Plate: 3.1 (5)).

Site-6 Veraval

Veraval is situated on the south Saurashtra coastline of the Gujarat. To commercial fisheries, Veraval is very important as it is one of India's largest fish landing centres. It collects huge quantities of waste from the port, including gas. It also collects from the city on a regular basis domestic sewage waste as well as heavy industrial waste and small-scale fish processing units. The exposure area of the intertidal zone varies from 30 meter to 50 meter during the low tide. The rocky shore line is interrupted by large channels and

boulders. The upper intertidal region is made up of sandy shores. The middle and lower intertidal zone is covered by a tide pool and deep crevices in which shallow tide pools are present in the middle intertidal zone while deep tide pools with small openings are available in the lower intertidal zone. The lower littoral zone of Veraval ends in a steep vertical drop to the sub-tidal zone. The intertidal zone of Veraval covers a distance of about 60- 90 m during the tides of spring. This intertidal zone is distinguished by numerous structural substratum variations such as bare rocky area, algal cover area, irregular rocky area with more pools and crevices which show their typical assemblage structure of benthic diversity (Fig. 3.4, Plate: 3.1 (6)).

Site-7 Sutrapada

Sutrapada is located 20 km south of Veraval. Gujarat is the leading manufacturer of NaHCO2 in India and nearly all of the NaHCO3 is produced by Gujarat Heavy Chemical Limited (GHCL) in Sutrapada. The fishermen colony and fish landing centres are placed on the eastern side. Sewage is discharged near the shoreline and the GHCL factory discharged is approximately 500 meters from the shore. The coastal areas of the site face moderate levels of anthropogenic activity. The intertidal area is rocky in nature with sandy shore at the upper intertidal zone. The slope of the Sutrapada shore is comparatively more than other study sites. The middle intertidal zone is fairly flat in nature, surrounded by shallow tide pools. In the lower part of the l mid intertidal zone there are large hard coral rocks. There are deep tide pools and deep interconnected channels and crevices in the lower intertidal zone. At low tide, these deep tide pools are submerged within water. The area of exposure of the intertidal zone at low tide varies from 100 meters to 140 meters. The water current and wind velocity partially support the movement of sediment towards the sea (Fig. 3.4, Plate: 3.1 (7)).

Site-8 Vadodra jhala

Vadodra Jhala is 30 km from Veraval, the nearest village on the Sutrapada-Kodinar highway. Inadequacy of information about geomorphic characteristics

on intertidal zone of Vadodra Jhala reflects the lack of exploration of this site. The rocky intertidal area consists of many small to large tide pools with a depth range of 0.2-0.8 m. Upper intertidal zone with multiple shallow tide pools originated from the cliff slopes and large rocks. Lower intertidal zone is a rocky flat area of lesser-quantity deep tide pool than the upper intertidal zone. The lower part of the intertidal mid zone ends with a steep slope of wide and deep tide pools and can be called a lower intertidal zone, often submerged during the low tide. Intertidal area of Vadodra jhala is exposed to approximately 50-60 m during low tides. Algal bed and seaweed bed coverage is found highest in this site compare to Sutrapada and Dhamlej. Crevices and tide pools provide enough protection from desiccation to allow a species to inhabit an area. Beneath the big rock, the sea anemone is suitable for hiding from predators aaand also less tolerating wave action (Fig. 3.4, Plate: 3.1 (8)).

Site-9 Dhamlej

Dhamlej is located 44 km south of Veraval. Dhamlej is a small fishing village. The village's coastal areas are mostly used by local people for fishing activities, and the study site detects a very low level of anthropogenic activity. The intertidal zone exposure area ranges between 100 meters and 120 meters during low tide. The intertidal zone is rocky in nature with a sandy shore in the upper zone. The intertidal zone between the upper and lower is smooth in nature. Very few shallow tide pools have been found in the mid-intertidal zone. Mostly small channels and crevices cover the mid-intertidal zone. The lower intertidal area is covered with tide pools that are shallow and wide (Fig. 3.4, Plate: 3.1 (9)).

Site-10 Kodinar (Mul Dwarka)

Kodinar is located 50 km south of Veraval, is a town famous for the Ambuja Cement Factory. The selected site is located in the village of Mul Dwarka, 3 km from the town of Kodinar. The intertidal zone consists of a hard-flat rocky substratum with small depressions that are interspersed with pools and puddles. The upper intertidal zone is made up of sandy shore with moderate

Slope. The upper intertidal zone consists of a gently sloping sandy shore. The upper part of the mid-intertidal zone is occupied by wide shallow tide pools, which during low tide remain filled with water while the lower part is protected by shallow rock crevices. Wide shallow tide pools cover the lower intertidal zone. There are also several wide tide pools that hold large hard coral rocks (Fig. 3.4, Plate: 3.1 (10)).

Site-11 Diu

Diu Island is an island off the southern coast of the Kathiawar peninsula of Gujarat, isolated by a tidal stream from the mainland Diu is bordered by Gujarat District of Gir-Somnath and Amreli in the north and by the Arab Sea on three sides. Diu is a tourist destination popular for its beaches and former colonial historical sites in Portugal. Nagoa and Jalandhar beaches were surveyed for Actiniarians Diversity based on their coast characteristics, tidal exposure and ecological exposure. Such as shallow sandy pools, rock pools and puddles, Zoanthid pits, rock caves and crevices (Fig. 3.5, Plate: 3.1 (11 a, b)).

Site-12 Alang

Alang is located 50km in southeast direction from Bhavnagar. Alang coastline has the rarest coastal geo-morphology, with the longest continental shelves, making it a geographically natural dry port, suitable for ship-breaking (Reddy et al., 2004). Alang is the world's largest ship breaking yard in Gujarat, annually scrapping hundreds of ships. The intertidal is separated preliminary as sandy, exposed rocky plus silty-clayey and rocky overlaid by silty-clayey mud. The upper intertidal area of study site consists of sandy shore, ranging in width from 20-30 m. Small rock boulders often obstruct the lower part of the sandy shore. The middle intertidal zone consists of rocky shore with muddy and sandy bottoms with shallow tide pools. The lower region has a relatively moderate layer of loose mud-clay with a rocky foundation. Depth of 50 tide-pool from all the three zones of intertidal zone were recorded and found to be 11.2 $\pm$ 5.51 cm. Intertidal area have heavy sedimentation load 5 $\pm$ 4.3 cm of depth (Fig. 3.6, Plate: 3.1 (12)).


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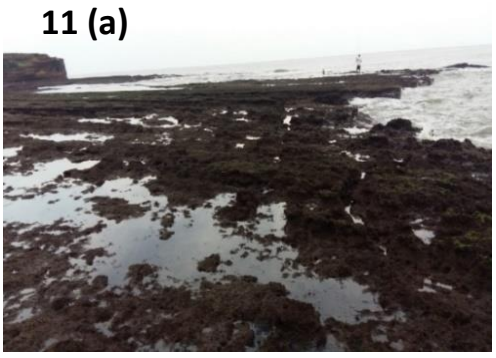

Plate 3.1: Habitats of study sites along the Saurashtra coast, Gujarat

(1) Okha (2) Mithapur (3) Shivrajpur (4) Dwarka (5) Mangrol (6) Veraval (7) Sutrapada (8) Vadodra jhala (9) Dhamlej (10) Kodinar (11 a) Diu, Nagoa beach (11 b) Diu, Jalandhar beach (12) Alang

3.1.4 STUDY DURATION

The proposed study was conducted from January 2015 to March 2019. Coastal survey was conducted extensively prior to this study. Rocky intertidal areas were identified and above-mentioned sites were selected for the work. Delineation of zone was carried out and study area was divided according to the specified littoral zones. Preferences of sea anemone were studied at microhabitat level. Study was carried out during the low tides by taking the advantage of large exposure of intertidal area. During each field visit the area was explored for the presence of new variants or associates of sea anemones. Sea anemones found bleached was regularly monitored on the monthly bases for recovery status.

3.2 MORPHOLOGICAL IDENTIFICATION OF SEA ANEMONES

3.2.1 SPECIMEN COLLECTION, PRESERVATION AND IDENTIFICATON

- *In situ* photography: Well focused photograph of fully exposed sea anemone was taken with clearly visible identifying characters such as: tentacles, oral disc, mouth, column of expanded anemone and verrucae Pattern. Photograph of retraced anemone was also taken for study.
- Collection of the specimen: The specimen was collected from the intertidal area during the low tide. Specimens were collected with their substratum using hammer and chisel. Care was taken not to destroy sensitive habitats. Buried anemones were collected using sieve as they were attached to stones or shells in the substratum and often moved down when disturbed. Anemones separated from substratum were collected using blunt instrument by separating base gradually from its hold. During the collection of specimens some important characters and reactions were noted: Tentacle morphology, stickiness of tentacles, strength of adhesion of the pedal disc to the substratum, adhesive of columnar structure, response to light, vibration and

disturbance in form of contraction. Specimen collected was labelled with field ID.

- Relaxation: It was adventurous to inject 3.5-7.5% MgCl2 or MgSO4 solution as anaesthetic *in situ* so that the anemone was relaxed during collection (England, 1987 and Moore, 1989). The specimens were relaxed before fixing in formalin to avoid contraction and mucus secretion during preservation. Specimens need less than one or several hours, depending on the species, until they are completely relaxed and do not respond to tactile stimuli.
- Fixation and Preservation: The relaxed animals were carefully transferred into a solution of 7 to 15% seawater: formalin (Concentration was varying according to the size of the specimen) (Stephenson, 1928 and Haussermann, 2004).
- Histological examination: The fixed specimens were cut longitudinally and embedded in paraffin. For histological characteristic studies, the sections were used to prepare longitudinal and cross (one type from each half) histological sections (5 µm thick) of the anemone body column were prepared. Sections were stained using Haematoxylin-Eosin (Stephenson, 1928; Carlgren, 1949 and Manuel, 1981). Histological sections were analysed to understand the mesenteries arrangements and different types of muscles under a light microscope.
- Identification: The identification of species was carried out on the bases of morphological and anatomical features using available morphological characteristics key.

3.2.2 MORPHOLOGICAL PARAMETERS OF SEA ANEMONES

During the collection of sea anemone some of the morphological parameters were recorded *in situ* measuring with the help of Vernier callipers are listed (Fig. 3.7):

- **Column Parameters**

1. CH = Column Height,
2. LD= Limbus Diameter

3. CD = Column Diameter
4. PDD= Pedal Disk Diameter
5. PDP= Pedal Disk Perimeter
6. PDA= Pedal Disk Area
    - **Oral Disc Parameter**
7. ODD= Oral Disc Diameter
    - **Tentacle Parameters**

For **Digiform type** of tentacle:

8. TTL= Total Tentacle Length

For **Bulbous type** of tentacle:

9. TTL = (tentacle length at maximum width as a proportion of total tentacle length)
10. LMW= Length at Maximum Width
11. WM= Maximum Width

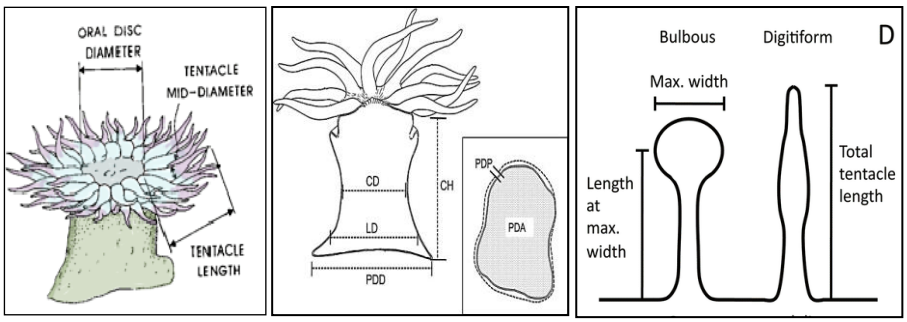

Figure 3.7 : Expanded specimen of sea anemone showing few of the morphological parameters

3.3 MOLECULAR IDENTIFICATION OF SEA ANEMONES

Collected samples were preserved in 95% to 100% ethanol. Tissue was taken from the Pedal disc to reduce the contamination with symbiont-DNA in zooxanthellae species.

3.3.1 DNA EXTRACTION

DNA extraction was performed using standard S. M Pinto *et al*., 2000 with modifications.

- Total genomic DNA was extracted from 70% ethanol preserved sea anemones. Approximately 20mg of tissue from the pedal disc were used, which avoided possible amplification of the zooxanthellae genome (Fautin and Smith, 1997).

- In all steps, shaking was for 15 min and centrifugation for 4 min at 6500 rpm, except in the third step that centrifugation was at 13,000 rpm. Prior to extraction the tissue fragments were placed in an eppendorf tube and incubated at 35ºC for 2hours in order to withdraw excess ethanol.

- In step one, small pieces of tissue were grounded in a final volume of 400 ml of lysis buffer [10smM Tris-HCL, pH 8.0, containing 0.25 M EDTA, pH 8.0, 2% (w/v) sodium dodecyl sulfate (SDS)].

- After incubation, an equal volume of phenol was added to the sample, which was then shaken and centrifuged. Approximately 360 ml of the supernatant solution was then transferred to a clean tube and an equal volume of phenol added. Shaking and centrifugation were performed using the same conditions as above and 350 ml of the supernatant was transferred to a new tube with an equal volume of Chloroform: Isoamylalcohol (24:1) and gently shaken before centrifugation.

- In step three, 300 ml of the supernatant was transferred to a new tube containing 30µl of 6 M NaCl and gently stirred. The DNA was precipitated with 2.5 volumes of very cold absolute ethanol and the samples centrifuged for 4 min at 13,000 rpm. The supernatant was drained off and the pellet was washed with 70% ethanol and air-dried for 24h with the tube inverted. The samples were re-suspended in an appropriate volume of Milli Q water and stored at -20˚C for complete solubility of DNA. The integrity of the extracted DNA was checked using 2 ml of each sample on a 1.0% Agarose gel stained with Ethidium bromide.

Determination of quality and quantity of DNA

(1) To evaluate the quality of the genomic DNA, gel analysis and Spectrophotometric method was carried out, A260/280 was measured and concentration of DNA was calculated using formula:

- 5µl of DNA sample was used to quantify the concentration of DNA by calculating the ratio of absorbance at 260nm and 280nm.
- The quantity of DNA can be checked as follows:

To take OD of DNA, take 5µl of DNA sample and 995µl of milli-Q water. (1 OD corresponds to 50µg/ml of DNA)

- Calculation:

Concentration of DNA= Absorbance at 260nm× Dilution Factor× 50µl/ml

Dilution factor = 200

(2) Agarose Gel Electrophoresis

Agarose Gel:

- 1.0gm Agarose powder dissolved in 100ml of 1X TAE Buffer.
- The integrity of DNA was confirmed by running the sample.

Solutions for Agarose Gel Electrophoresis:

Running Buffer

- 50X Tris Acetate EDTA (TAE) Buffer
- 242-gram Tris base, 57.1 ml Glacial Acetic Acid, 100ml of 500mM EDTA (pH-8.0) dissolve in final volume of 1 litre.

Ethidium Bromide

- Stock solution having concentration of 10mg/ml was prepared using distilled water and stored in dark (Brown) coloured vial.

DNA Loading Dye

- Loading dye was prepared by mixing 3ml glycerol (30%), 25mg Bromophenol blue (0.25%) and dH2O to 10ml.

Plate preparation and casting the gels

Agarose gel casting plate and comb were wiped with isopropanol. The comb was placed in the given slits of the plate. Calculated amount of Agarose in TAE buffer was mixed to prepare 0.8% Agarose gel. The Agarose

was dissolved completely in the buffer by heating the mixture at 80-85˚C and was cooled to 50˚C. Ethidium bromide was added in a final concentration of 10mg/ml and mixed well. Liquid was gently poured into the casting tray before it gets solidified. The comb was removed slowly after complete solidification of the Agarose gel.

Preparation of samples and scanning of gels

The DNA and PCR product samples approximately 5µl volume were mixed with 1µl gel loading dye and were carefully loaded in the wells using gel-loading tips. Electrophoresis was carried out at 100V. The gel images were recorded in JPEG and TIF formats using gel documentation system.

3.3.2 POLYMERASE CHAIN REACTION

- PCR was carried out using 2X final concentration of One Taq® Standard Buffer and, template DNA (50ng/µl). The reaction was carried out in Thermal cycler.
- Final concentration of PCR reagents in reaction mixture (20µl): One Taq® Standard Buffer Mix (10µl), 100 pmole of each Primer (2µl each), 50-100ng Template (2µl each), MilliQ (4µl)

Primer	Primer Sequence
NS1	5'- GTAGTCATATGCTTGTCTC-3'
NS4	5'- CTTCCGTCAATTCCTTTAAG -3'

Table 3. 1 : Primers used for 18s Marker

Stage 1 x 1 Cycle	**Stage 2** x 35 Cycles	**Stage 3** x 1 Cycle
Initial Denaturation 95˚C -3 min	Denaturation 95˚C – 30 sec	Final Extension 72˚C - 5 min
	Annealing 50˚C – 30 sec	
	Extension 72˚C – 90 sec	

Table 3. 2 : PCR Condition

Purification of the PCR Product

PCR amplified products were analysed on 2% Agarose gel. Positive amplified products were purified using the ExoSAP-IT PCR purification Kit, according to the manufactured instructions. In a positive PCR product, 4µl of ExoSAP were added. An Applied Biosystems (Veriti) ® thermal cycler was run.

Stage 1	Stage 2	Stage 3
37◦C	80◦C	4◦C
15 mins	15 mins	∞

Table 3. 3 : PCR purification condition

3.3.3 SANGER SEQUENCING

Sanger sequencing is a method of DNA sequencing first commercialized by Applied Biosystems, based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication Developed by Frederick Sanger and colleagues in 1977.

The classical chain-termination method requires: a-single-stranded DNA template, b-a DNA primer, c-a DNA polymerase, d-normaldeoxynucleosidetriphosphates (dNTPs), e-modified di-deoxynucleotidetriphosphates (ddNTPs), the latter of which terminate DNA strand elongation. These dNTP's and ddNTP's lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to stop extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labelled for detection in automated sequencing machines.

The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while the other added nucleotides are ordinary ones. The dideoxynucleotide is added to be approximately 100-fold lower in concentration than the

corresponding deoxynucleotide, allowing for enough fragments to be produced while still transcribing the complete sequence. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments are heat denatured and separated by size using gel electrophoresis. This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, and C). The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image.

Sequence validation and phylogenetic analysis

The sequence was validated and specific contigs of anemones were obtained using BioEdit 7.0.5.3. NCBI BLAST was used to check the genetic relationships of sequences with other sea anemone species. Furthermore, it was subjected to tree construction using statistical neighbourhood joining Distance where the test of phylogeny was performed.

3.4 THERMAL STRESS IMPACT ON SEA ANEMONES

3.4.1 SEA ANEMONE BLEACHING AND SEA SURFACE TEMPERATURE (SST)

The study of bleaching was carried out covering two distinct seasons viz., summer and post monsoon. The survey was conducted in the intertidal areas of the sites during low tides. The coordinates were recorded with E-trex Garmin hand held GPS navigator. The observations include the record of affected Scleractinian species and Actiniarians species. The SST data have been derived from the www.oceanmotion.org and www.esrl.noaa.gov for the annual and monthly mean SSTs of the Gulf of Kachchh.

3.4.2 HSPs GENE EXPRESSION STUDY OF SEA ANEMONE UNDER THERMAL STRESS

Sampling of *Urticina cledenstina* in different intertidal conditions

To examine possible effects of low tide on the expression of HSP60 and HSP70, specimens were collected from tidal pools during extreme low tides. The sampling was done from different deapths of the same tidepool situated at vadodra jhala site of Saurshtra coast. Samples were collected from upper and lower parts of the tidepool when the Sea Water Temperature (SWT) varies between 20°C-31°C and examined their levels of HSP60 and HSP70 expression. Pieces of tentacle crown, oral disk, column, and pedal disk were dissected, immediately frozen in liquid nitrogen, and stored at -70°C.

Total RNA Isolation

Total RNA was isolated by Trizol Invitrogen according to the method of Peterson and Freeman (2009). The cells were harvested and pellet down at 4000rpm for 10 min. Supernatant were discarded and were re-suspended in 500µl TRIzol reagent (15596-026, Invitrogen, USA). The homogenate was taken into 2 ml micro centrifuge tubes (Tarsons, India). After successful homogenization, equal volume of Trizol reagent was added. For complete dissociation of nucleoprotein complexes, homogenized samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of chloroform and was vigorously shaken for effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes till the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000x g for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, an inter-phase, and a colourless upper aqueous phase. An aliquot of upper aqueous phase was then transferred into a new 1.5 ml micro centrifuge tube using 1000µl pipette. Precipitation was done by adding 500µl of isopropanol to the supernatant that was transferred. The samples were kept in -20°C for 10 minutes, centrifuged at 12,000x g for 15 minutes at 4°C. After precipitation, the supernatant was

discarded without disturbing the pellet and was washed in 1000 µl of 75% ethanol and 300µl of absolute ethanol was added to the pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 x g for 7 minutes at 4°C. The pellet was re-suspended by adding 50µl of DEPC water (Diethylpyrocarbonate) and was incubated at room temperature for 10 mins.

cDNA Synthesis

After the purity check of RNA that was validated using spectrophotometer, 1µg of total RNA was used for reverse transcription reaction using verso cDNA synthesis kit (Thermo Scientific-AB-1453/A). Briefly, fresh nuclease free PCR tubes (Tarsons, India) were taken, in which 4µl of 5X cDNA synthesis buffer, 2µl dNTP mix, 1µl of RNA primer (oligonucleotides), 1µl of RT enhancer, 1µl of verso enzyme mix, 1-2µl of RNA template (according to the spectroscopic quantification i.e. 1 ng) and the final assay volume was made to 20µl using nuclease free water. The tubes were effectively mix by giving a short centrifuge spin for 30s at around 2000 x g. The tubes containing the kit mixture was PCR amplified by 2 step reaction process. Firstly, the 1 cycle of cDNA synthesis was carried out at 42˚C for 30 mins followed by 1 cycle of inactivation at 95˚C for 2mins.

RT-PCR Amplification

Quantitative RT-PCR was performed using SYBR select Master Mix (Applied Biosystems) in Quant Studio 12K (Life technology) real-time PCR machine with primers to detect selected messenger RNA (mRNA) targets. The melting curve of each sample was measured to ensure the specificity of the products. The mean of housekeeping gene β-actin was used as a control to normalize the variability in the expression levels and data was analyzed using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Following Primers pairs were used for qPCR:

Accession No.	Gene Name	Sequence	Amplicon size (bp)
isotig08469	HSP90	Fw CCGATGCTTTGGACAAGATTC Rv ATGTCAGCCTTGGTCATTCC	**151**
isotig04537	HSP70	Fw ACAAGAGGGCAGTCAGGAGA Rv TTTGCCCAACTTGGAGTCAC	**218**

Table 3. 4 : Primer details used for RT-PCR