Chapter 4

Diatoms in the epibiotic community
4.1 INTRODUCTION

In densely populated marine environments, space is often a limiting resource in epibenthic communities (Jackson and Buss 1975). When free space is limiting, living substrata may become important for epibiosis. In the marine environment, a variety of benthic plants and animals form hard substrates in soft-bottom sediments. Epibiosis of such substrata (i.e. non-symbiotic, facultative association between epibionts and basibionts) becomes a highly valuable phenomenon for the survival of sedentary organisms (Wahl 1989). Some organisms appear to tolerate a considerable load of epibionts (Rützler 1970, Davis and Wright 1989). Epibionts on the hosts have been indicated to have a protective value for the host via camouflage (Ingle 1983, Rasmussen 1973). Only certain biological surfaces resist colonization to variable degrees for more or less extended periods (Fletcher and Marshall 1982). Control of epibiosis by the host organisms is achievable by one or any combination of ecological, mechanical, physical and chemical factors. Though there is considerable literature on the mechanisms by which invertebrates deter or shed fouling organisms, there is very little data on natural levels of fouling in invertebrate communities (Davis and White 1994).

Horseshoe crabs act as moving substrata for simple to complex communities of small marine organisms. Horseshoe crabs carry a variety of epibionts on their external surface, including green algae, diatoms, coelenterates, flatworms, mussels, oysters, annelids, barnacles, tunicates, bryozoans, isopods, amphipods, gastropods, pelecypods and polychaetes (Humm and Wharton 1942, Roonwal 1944, Rao and Rao 1972, Davis and Fried 1977, Mackenzie 1979, Shuster 1982, Jeffries et al. 1989, Saha 1989, Debnath 1992, Key et al. 1996). However, taking into account that adults reach terminal anecdyisis once sexually mature and live with their carapace for 4 to 9 years, the intensity of epibiosis appears negligible. Mikkelsen (1988) also observed that barnacles are usually
seen on males, indicating a potential gender difference. In this investigation an attempt has been made to explore the gender and spatial differences in the epibiosis of the horseshoe crab *Tachypleus gigas*, chemical defense mechanism to control epibiosis and to postulate the possible causative factors.

Horseshoe crabs live in moderately deep waters and migrate to near shore waters for breeding. When approaching the beach to nest, a female is almost always accompanied by a male clasping her with his modified claws (Cohen and Brockman 1983). In India, the horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda* are found confined to Orissa and West Bengal coasts. Along the Orissa coast, they are found near Burhabalanga estuary and Abana. *T. gigas* is the most abundant of the two species (Vijaykumar 1992, Chatterji 1994).

4.2 MATERIALS AND METHODS

Living specimens of *Tachypleus gigas* were collected near the Burhabalanga estuary, Orissa coast (Fig. 4.1) in March and August of 1997, and again in February and August of 1998. The horseshoe crabs used in the study were haphazardly collected, amplexed pairs so that all crabs were sexually mature and in terminal anec dysis. The specimens were collected during receding high tide and were transported to the laboratory in seawater for the evaluation of diatoms and macro-epibionts. The horseshoe crabs collected were sexed. The length and width of the prosoma were measured and were used as standard morphometric proxies to determine the size of the horseshoe crabs. Since the carapace (dorsal side) was uneven, the total area was measured by marking it into different geometric figures like triangles and trapeziums (a quadrilateral with only one pair of sides parallel) and summing their areas. The dimensions of the geometric figures differed for
different crabs depending on the size of the carapace. Surface pH of live crabs was
determined (six times each for male and female carapaces) at the collection site by
placing pH indicator paper (Qualigens, pH 1 to 14) on humid carapaces. Surface
wettability of 14 air-dried carapaces (seven each for male and female carapaces) was

Fig. 4.1. a. Location of the sampling site where the horseshoe crabs *Tachypleus
gigas* frequently migrate to the shore for breeding. b. Area of the sampling
locality marked in a.
determined by the drop-spread method as described by Gerhart et al. (1992), using HPLC-grade water and methanol. The spread of 25 μl drops was measured using a series of solutions of 100, 80, 60, 40, 30, 20, 10 and 0% methanol and calculating the standardized harmonic mean (SHM) of the diameter (mm) of the drop. The SHM values strongly correlate with the combined polar components of surface wettability (Gerhart et al. 1992). Using polar solutions of water and methanol, the drop-spread method can detect changes in the polar characteristics of different surfaces with a sensitivity that approaches that of measurements obtained with a contact angle goniometer (Gerhart et al. 1992). The polar solutions used in the drop-spread method also allowed non-destructive testing of the surfaces (Gerhart et al. 1992).

4.2.1. Enumeration of diatoms

Specimens (two pairs in March 1997, three pairs each in August 1997, February 1998 and August 1998), free of macro-epibionts, were collected for the evaluation of diatom communities. Quantification of diatoms associated with horseshoe crab carapaces was carried out by scraping the entire carapace with a nylon brush into a known volume of filtered seawater. The scraped material was preserved in Lugol's iodine, and its diatom flora was enumerated by the sedimentation technique (Hasle 1978). The diversity (Shannon-Wiener diversity index, H') and evenness (J') of the diatom community was evaluated. The log (x + 1) transformed values of diatom abundance were further analyzed using cluster analysis. The dissimilarity levels were measured through squared Euclidean distance and group average method as described by Pielou (1984). The log-transformed values of abundance of all diatoms (cells dm⁻²) were subjected to two-way analysis of variance (ANOVA), with unequal but proportional subclass numbers (Sokal and Rohlf 1981), for evaluating the differences with respect to sampling period, sex and the interaction of sampling period and sex (eight subgroups; four samples for each sex).
4.2.2. Enumeration of macro-epibionts

In the evaluation of macro-epibionts, 6 pairs of crabs were used in March 1997, 15 pairs in August 1997, 15 pairs in February 1998 and 10 pairs in August 1998. The macro-epibiont populations from the dorsal sides of the carapaces (prosoma and opisthosoma) were enumerated. Solitary forms like barnacles (*Balanus amphitrite*, Darwin), false oysters (*Anomia* sp.) and sea anemones were counted and represented in terms of individual counts per square decimeters. Area covered by barnacles and bryozoans was determined in terms of percentage coverage. Dimensions of the barnacles, i.e. basal rostro-carinal and latero-lateral diameters in millimeters, were measured by using a Vernier caliper to calculate the basal area. Basal diameter (mm) of barnacles was considered as a measure of size to determine the growth of barnacles. The individual basal area was used in evaluation of total area covered by barnacles. Size-frequency distribution was evaluated by grouping barnacles in 2-mm intervals (individuals <2 mm were considered spats). Mapping of the macro-epibiont distribution on carapaces was carried out by marking the carapaces into different zones based on the zones exposed during the nesting period in the nearshore waters (Fig. 4.2). The prosoma was categorized into three regions: (1) the cardiopthalmic ridge (Prs1), (2) the anterior prosoma up to the level of lateral eyes (Prs2) and (3) the two flanks of the prosoma (Prs3). The opisthosoma was categorized into two regions: (1) the anterior (uncovered region; Opt1) and (2) the posterior (covered region; Opt2). The arcsine transformed values of the total macro-epibiont; barnacle and bryozoan percentage coverage’s were separately subjected to two-way analysis of variance (ANOVA) with replication (Sokal and Rohlf 1981). This was done in order to understand the differences associated with different regions of the carapace (prosoma and opisthosoma) versus those associated with gender. These were analyzed further using cluster analysis, to better understand the
dissimilarity pattern. The dissimilarity level was measured through squared Euclidean distance and the group average method as described by Pielou (1984).

![Diagram of Tachypleus gigas carapace with demarcated regions]

Fig. 4.2. Tachypleus gigas Carapace demarked for epibiosis evaluation (Pr prosoma: Prs1 cardiopthalmic region, Prs2 anterior prosoma up to the level of lateral eyes, Prs3 flanks of the prosoma; Opt opisthosoma: Opt1 anterior opisthosoma (uncovered region)).

4.2.3. Scanning electron microscopy (SEM)

The different regions of male and female horseshoe crab carapaces (dorsal side) were examined by SEM to evaluate the surface characteristics. Replicate samples from different regions (Prs1, Prs2, Prs3, Opt1 and Opt2) of the carapace were mounted on stubs, gold-coated and examined at 15 kV with a JEOL JSM-5800 LV scanning electron microscope. The observations were repeated with three male and female specimens.

4.2.4. Extraction of Horseshoe crab secretion

The animals were starved for 24 h prior to the commencement of the experiment. Male and female horseshoe crabs were kept separately in tanks (2 each) containing 24 liters of
GF/F filtered seawater at room temperature (29 ±2 °C; Salinity: 34 psu) whereas, in the third tank only seawater was kept and treated as experimental control. After 60 h, animals were removed from the water and water samples including control were eluted through a column packed with Amberlite-XAD-2 resin. Amberlite-XAD-2 resin column was washed with distilled water to remove the salts. The organic compounds adsorbed on the XAD-2 resin were extracted using organic solvent methanol (AR grade). The eluted methanol was evaporated using a rotary vacuum evaporator at 40 °C to get dried extract. The concentrated extracts were used for bioassay studies with fouling diatoms.

4.2.5. Diatom assay

The diatoms (Amphora coffeaeformis and Navicula transitans var. derasa f. delicatula) dominant in micro-fouling communities were isolated from the intertidal sediments of Dona Paula Bay, west coast of India (15° 27’ N and 73° 48’E). The isolated diatoms were maintained as pure cultures in f/2 medium (Guillard and Ryther 1962).

The experiment was carried out in polystyrene multi-wells (corning #25810) at a constant temperature of 20±1 °C in an incubator with 12:12 h light and dark photoperiod. The secretion of horseshoe crab was reconstituted with methanol (AR) at a rate of 400 µg in 2 ml of methanol. The assay protocol included evaluation of the female and male horseshoe crab extracts at a concentration of 50 µg/well. This was achieved by introducing 250 µL of the reconstituted extract to the multiwells. In order to evaporate methanol content of the extract, the multiwells were kept under sterile condition in a laminar flow chamber for 3 to 4 h. A solvent control and seawater control was assayed simultaneously. The assay wells were inoculated with ~ 50,000 diatom cells in 5 ml sterilized filtered seawater enriched with f/2 medium at a salinity of 34 psu. Cells adhering to the bottom of the wells were quantified through light microscopy as abundance mm⁻². In each of these
 assay conditions, enumeration of the diatom numbers was carried out at an interval of 24, 48 and 72 h.

4.2.6. Chromatographic study of crude methanol extract of the secretions of horseshoe crab

Thin layer chromatography of concentrated secretions of male and female horseshoe crabs and experimental control (seawater) was carried out on commercial silica gel coated TLC plate (Merck Cat. No. 1. 05554. 001). The 25% acetone/petroleum ether was used as a solvent system for running the plates. The plate was developed by spraying anisaldehyde-sulphuric acid spray (1 ml anisaldehyde + 1 ml Conc H$_2$SO$_4$ + 18 ml ethyl alcohol) and heated at 110° C. Number of spots was observed and RF (Resolution factor) value of each spot was measured.

4.3 RESULTS

As with all the horseshoe crabs, females were larger than males (Fig. 4.3). The surface area of the carapace of female horseshoe crabs was 55 to 60% more than that of males.

![Fig. 4.3. Tachypleus gigas.](image)

**Fig. 4.3. Tachypleus gigas.**
Prosome size of male and female horseshoe crabs used in the study.
The surface area of the prosoma was approximately 75% greater than the opisthosoma in both sexes. The surface area of the female's prosoma and opisthosoma was 55 to 65% greater than that of the males.

4.3.1. Surface properties of the carapace

The whole body is covered by tough chitinous exoskeleton, which is sage green in color. The males were lighter in color than the females. The results obtained for surface wettability indicated the male carapace to be slightly more hydrophobic (SHM = 14.5 ± 2.34) than the female carapace (SHM = 18.7 ± 1.8). The surface pH did not vary much between male and female carapaces and ranged from 8 to 8.5.

4.3.2. Scanning electron microscope (SEM)

Electron micrographs of the carapace of male and female horseshoe crabs are shown in Figs. 4.4a and b, respectively. SEM revealed that the male carapace was comparatively

![Male carapace](image1.png)

![Female carapace](image2.png)
rough as compared to the smooth carapace of females. Star-shaped openings were observed on the female carapace in the cardiophthalmic region and posterior part of the rim surrounding the cardiophthalmic (posterior prosoma) region (Fig. 4.4b, Prs1 and Prs2). Such openings were not seen on any of the male carapaces.

4.3.3. Diatoms

Diatoms recorded from male and female horseshoe crabs belonged to 20 (11 pennates and 9 centrics) and 17 (10 pennates and 7 centrics) genera, respectively. The diatom abundance (cells dm$^{-2}$), generic diversity ($H'$) and evenness values ($J'$) were lower for females than for males (Figs. 4.5a, b).
Fig. 4.5. *Tachyleus gigas*. A comparison of a diatom cell abundance (cells dm\(^{-2}\) X 10\(^3\)), b diatom diversity (H') and evenness (J') and c ratio of pennates/centrics in male and female horseshoe crabs during different occasions. Error bars indicate standard deviation.
Pennate diatoms dominated the epibiotic community (Fig. 4.5c). Their dominance in the epibiotic community ranged from 31 to 90% in males and 52 to 93% in females. Among the pennates *Navicula* spp. and *Nitzschia* spp. and among the centrics *Skeletonema* sp. were dominant. Two-way ANOVA of diatom abundance (cells dm$^{-2}$) revealed that there is a significant variation between the sexes and the sampling period (Table 4.1). Interaction of sampling period and sexes revealed insignificant variations, indicating influence of sampling period to be equal in both the sexes (Table 4.1).

**Table 4.1. Tachypleus gigas.** Results of two-way ANOVA with uneven sample sizes comparing diatom density (cells dm$^{-2}$) between sexes (male and female) over period of sampling.

<table>
<thead>
<tr>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>$F_s$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgroups (8; 4 samples for each sex) 7</td>
<td>5.9</td>
<td>0.8</td>
<td>6.5</td>
<td>$&lt; 0.005$</td>
</tr>
<tr>
<td>Sampling period (4) 3</td>
<td>4.1</td>
<td>1.4</td>
<td>10.4</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Sexes 1</td>
<td>1.6</td>
<td>1.6</td>
<td>12.4</td>
<td>$&lt; 0.005$</td>
</tr>
<tr>
<td>Sampling period X sexes 3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Error 14</td>
<td>1.8</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 21</td>
<td>7.7</td>
<td></td>
<td></td>
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</tbody>
</table>

Generic clustering of the diatom population revealed that cluster I comprising of *Navicula* spp., *Nitzschia* spp. and *Skeletonema* sp. were the most dissimilar forms encountered in both males and females (Figs. 4.6a, b). However, in the case of males these three forms merge with the rest of the community at a greater dissimilarity level (63.5), whereas in the case of females, the merger was complete at lower dissimilarity level (34).

### 4.3.4. Macro-epibionts

Macro-epibionts consisted of coelenterate *Metridium* sp. (sea anemone), the bryozoan *Membranipora* sp., the barnacle *Balanus amphitrite* (Darwin) and the bivalves *Anomia* sp. (false oyster) and *Crassostrea* sp. Taxon frequency and diversity of macro-epibionts were determined on the carapaces (dorsal side) of live specimens during the periods of
Fig. 4.6. *Tachyleus gigas*. Generic cluster dendograms of the epibiotic diatom community from **a.** male and **b.** female horseshoe crab.
sampling. Acorn barnacles (*B. amphitrite*) and encrusting bryozoans (*Membranipora* sp.) were the most abundant forms encountered in terms of coverage (Fig. 4.7).

Coverage of macro-epibionts was greater on male carapaces than on female carapaces (Fig. 4.8a). The macro-epibiont coverage was greater on the opisthosoma than prosoma (Figs. 4.8b, c). The telson of the horseshoe crab was free of macro-epibionts. The total area covered by macro-epibionts (which includes all forms recorded), when subjected to two-way ANOVA, revealed that there is a significant variation between the genders and between the prosoma and opisthosoma of the carapace (Table 4.2). Cluster analysis revealed the total macro-epibiont coverage to be different between the sexes. The least dissimilarity was seen in the case of female prosoma and opisthosoma (Fig. 4.9a).
Fig. 4.8. *Tachypleus gigas*. Comparison of total macro-epibiont coverage between male and female horseshoe crabs on a. the whole carapace, b. the prosoma and c. the opisthosoma during different sampling periods. Error bars indicate standard deviation.
Fig. 4.9. *Tachyleus gigas*. Dendograms of the a. total macro-epibiont coverage, b. barnacle coverage and c. bryozoan coverage to compare gender differences and between the prosoma and opisthosoma.
Table 4.2. *Tachypleus gigas*. Results of two-way ANOVA comparing macro-epibiont coverage between sexes (male and female) and between different parts (Prosoma and Opisthosoma) of carapace (*Prs* Prosoma; *Opt* Opisthosoma).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>( F_s )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parts (Prs &amp; Opt)</td>
<td>1</td>
<td>189.9</td>
<td>189.9</td>
<td>14.6</td>
<td>( &lt; 0.001 )</td>
</tr>
<tr>
<td>Sexes</td>
<td>1</td>
<td>3555.4</td>
<td>3555.4</td>
<td>27.4</td>
<td>( &lt; 0.001 )</td>
</tr>
<tr>
<td>Parts X Sexes</td>
<td>1</td>
<td>712.4</td>
<td>712.4</td>
<td>5.5</td>
<td>( &lt; 0.050 )</td>
</tr>
<tr>
<td>Error</td>
<td>180</td>
<td>23394.4</td>
<td>129.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>183</td>
<td>27852.1</td>
<td></td>
<td></td>
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</tbody>
</table>

Barnacle abundance (ind dm\(^{-2}\)) and coverage was also greater on the male carapaces than on the female carapaces (Fig. 4.7). Barnacle coverage was less on the prosoma than on the opisthosoma in both the sexes (Fig. 4.10). ANOVA revealed a significant difference in barnacle coverage between the sexes and between the prosoma and opisthosoma of the carapace in both the sexes (Table 4.3). Cluster analysis of barnacle coverage showed a pattern similar to that of total macro-epibiont coverage, whereby the sexes separated out as different clusters. Male opisthosoma was the most dissimilar form, followed by male prosoma, and the least dissimilar were observed in the cases of female opisthosoma and prosoma (Fig. 4.9b). Mapping studies revealed that the barnacle distribution (both adults and spat) on male and female carapaces was not uniform. In the case of females, no barnacles were found on the rim surrounding the cardiopthalmic region (i.e. the anterior
portion of the prosoma which is subjected to mechanical abrasions) and in the posterior area of the opisthosoma (i.e. the area covered by males) (Table 4.4). The abundance of barnacles was highest in the rough zone (Prs1 and Opt1) of both female and male carapaces. Barnacle abundance was less on female carapace than on male carapace during all the sampling periods. Fewer barnacles in the 6 to 8 mm size range (reproductive size of Balanus amphitrite) were seen on females than on males (Fig. 4.11). The size-frequency distribution of barnacles on female and male carapaces reveals that the recruitment of larvae on the carapace is high, but few recruits survive to maturity (as indicated by size) (Fig. 4.11).

Encrusting bryozoans were the other dominant organisms contributing to total macro-epibiont coverage (Fig. 4.7). The bryozoan coverage was greater in the case of males than females (Fig. 4.7). Encrusting bryozoan coverage was found to be less on the prosoma than on the opisthosoma in both sexes (Fig. 4.12). Coverage of encrusting bryozoans did not vary significantly between the sexes, but was significantly different between the parts of the carapace (Table 4.5). Cluster analysis for bryozoan coverage revealed less dissimilarity in comparison to total macro-epibiont coverage and barnacle coverage. The least dissimilarity was found among regions rather than sexes (Fig. 4.9c).

Table 4.4. Tachypleus gigas. Intensity of macro-epibiont distribution on marked areas of carapace. Observations from 46 carapaces for each sex during different sampling periods, March 1997 to August 1998. Numbers relate to the number of individuals in the case of barnacles and percentage occurrence in the case of bryozoans (M male; F female; Prs1 cardiophthalmic region; Prs2 anterior prosoma up to the level of lateral eyes; Prs3 flanks of the prosoma; Opt1 anterior opisthosoma (uncovered region); Opt2 posterior region (covered region).

<table>
<thead>
<tr>
<th></th>
<th>Prs1</th>
<th>Prs2</th>
<th>Prs3</th>
<th>Opt1</th>
<th>Opt2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M/F</td>
<td>M/F</td>
<td>M/F</td>
<td>M/F</td>
<td>M/F</td>
</tr>
<tr>
<td>Barnacle spat</td>
<td>12±58</td>
<td>2±58</td>
<td>1±5</td>
<td>0.2±2</td>
<td>0.2±1</td>
</tr>
<tr>
<td>Adult</td>
<td>6±11</td>
<td>1±2</td>
<td>4±8</td>
<td>0.1±1</td>
<td>1±3</td>
</tr>
<tr>
<td>Bryozoa (%)</td>
<td>15</td>
<td>2</td>
<td>24</td>
<td>14</td>
<td>33</td>
</tr>
</tbody>
</table>
Fig. 4.10. *Tachypleus gigas*. Comparison of *Balanus amphitrite* a, b % cover and c, d abundance (ind dm\(^2\), of different size groups) between male and female horseshoe crabs on a, c prosoma and b, d opisthosoma during different sampling periods. Error bars indicate standard deviation.

Table 4.5. *Tachypleus gigas*. Results of two-way ANOVA comparing bryozoan (*Membranipora* sp.) coverage between sexes (male and female) and between different parts (prosoma and opisthosoma) of carapace (*Prs* Prosoma; *Opt* Opisthosoma; *ns* not significant).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>SS</th>
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<th>(p)</th>
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</thead>
<tbody>
<tr>
<td>Parts (Prs &amp; Opt)</td>
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<td>234.9</td>
<td>234.9</td>
<td>6.60</td>
<td>≤ 0.01</td>
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<td>Sexes</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.01</td>
<td>ns</td>
</tr>
<tr>
<td>Parts X Sexes</td>
<td>1</td>
<td>39.4</td>
<td>39.4</td>
<td>1.10</td>
<td>ns</td>
</tr>
<tr>
<td>Error</td>
<td>180</td>
<td>6441.5</td>
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<td></td>
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<tr>
<td>Total</td>
<td>183</td>
<td>6716.3</td>
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</table>
Fig. 4.11. *Tachypleus gigas*. Size frequency distribution of *Balanus amphitrite* on male and female horseshoe crabs during different sampling periods (*arrows* indicate sexually mature size class).
4.3.5. Diatom assay

4.3.5.a. Amphora coffeaeformis

The average number of cells in the presence of female horseshoe crab extract was found to be minimum, 29 cells $\text{mm}^{-2}$ after 24 h and 118 cells $\text{mm}^{-2}$ after 72 h (Fig. 4.13a). The maximum number of cells was seen in control and rearing seawater wells. In control wells after 24 h, 101 cells $\text{mm}^{-2}$ and after 72 h, 1156 cells $\text{mm}^{-2}$ were observed (Fig. 4.13). The number of cells in the male extract was 74 cells $\text{mm}^{-2}$ after 24 h and 281 cells $\text{mm}^{-2}$ after 72 h (Fig. 4.13). This investigation revealed that the female horseshoe crab extract inhibited the multiplication of $A. \text{ coffeaeformis}$. 
4.3.5.a. *Navicula transitans* var. *derasa f. delicatula*

The results obtained for 24 h, 48 h and 72 h observations indicated that there was not much significant difference in the number of cells among the female secretion (44 to 210 cells mm\(^{-2}\)), male secretion (29 to 255 cells mm\(^{-2}\)), experimental control (47 to 243 cells mm\(^{-2}\)), methanol control (287 to 949 cells mm\(^{-2}\)) and sea water control (48 to 371 cells mm\(^{-2}\)) (Fig. 4.13). This experiment indicated that the secretion from horseshoe crab has no influence on this test organism.

**Fig. 4.13.** *Tachypleus gigas*. Influence of concentrated secretions of horseshoe crab on diatom growth (FHCS: Female horseshoe crab secretion, MHCS: Male horseshoe crab secretion, EC: Experimental control, SC: Solvent control, C: Control (sterilized sea water).
In general, diatom assays revealed that female horseshoe crab secretions inhibited the multiplication of *A. coffeaeformis*. However, normal growth was observed with male horseshoe crab secretions and controls. In case of *N. transitans var. derasa f. delicatula* the growth was normal with both male and female horseshoe crab secretions and controls. Field observations of diatom flora on male and female horseshoe crabs revealed that *Navicula* spp. was the most dominant form among the diatoms encountered in the community. This study showed that the secretion of female horseshoe crabs has the potential for the control of species-specific epibiotic diatoms.

4.3.6. Chromatographic study

The RF values of the spots for male and female horseshoe crab extracts and experimental control obtained from TLC plates are tabulated in Table 6. TLC plate indicated that female horseshoe crab extract had 4 spots whereas; male and experimental control appeared to have three similar major spots (Fig. 4.14).

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>FHC</th>
<th>MHC</th>
<th>EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.193</td>
<td>0.204</td>
<td>0.182</td>
</tr>
<tr>
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<td>0.636</td>
</tr>
<tr>
<td>3</td>
<td>0.761</td>
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</table>

Fig. 4.14. *Tachypleus gigas*. Thin Layer Chromatographic (TLC) plate (M: male horseshoe crab secretion, F: female horseshoe crab secretion, C: experimental control.)
4.4 DISCUSSION

Horseshoe crabs are slow-growing animals having a life span of 15 to 18 years. Their development to sexual maturity requires 9 to 10 years for males and 10 to 11 years for females (Mikkelsen 1988). After attaining sexual maturity crabs are in terminal anecydysis; the carapace covering the body is not shed. In spite of this, the carapace of the horseshoe crab is comparatively free of epibionts. In an environment where substratum availability is sparse in soft-bottom sediments, these hard-shelled organisms may serve as suitable substrata for epibionts.

In this investigation, we explored the differences in the epibiotic community of *T. gigas*, according to gender. The dominant diatom forms were *Navicula* spp., *Nitzschia* spp. and *Skeletonema* sp. in both the sexes (Figs. 4.6a, b). However, there were significant differences between the sexes in diatom abundance and diversity. The abundance, diversity and evenness values of diatoms were lower for females than for males (Figs. 4.5a, b). Among the macro-epibionts, barnacles (*B. amphitrite*) and bryozoans (*Membranipora* sp.) were dominant on the carapaces of both the sexes (Fig. 4.7). Both of these organisms are important constituents of the macrofouling community in Indian waters (Karande 1965; Anil 1986). There were significant differences in coverage of macro-epibionts between the male and female carapaces. Total macro-epibiont coverage and coverage of barnacles and bryozoans were also lower on the carapace of female crabs.

The size-frequency distribution of barnacles indicated that settlement does take place in high numbers, but few barnacles remain attached until reproductive age. This conclusion is drawn from the observation made earlier that *B. amphitrite* attains sexual maturity at a size (basal diameter) of 7.3 mm. *B. amphitrite* reaches this stage in about 20 to 22 d
under normal submerged conditions (Iwaki and Hattori 1987; authors' personal observations). The arrow marks in Fig. 4.11 indicate the abundance of barnacles that are in this size group in the epibiont community of the horseshoe crab. Here again, the larger barnacles were greater in abundance on the male carapace. Mapping of the macro-epibiont population belonging to this group also indicated considerable differences with different regions of the carapace (Fig. 4.2; Table 4.4). For both barnacles and bryozoans, the macro-epibiont coverage on the prosoma is less than on the opisthosoma. Differences in the structure of the epibiont community between sexes or among regions of the carapace may be related to: (1) changing habitat, (2) mechanical abrasion and surface availability, (3) requirements of epizootic larvae and/or (4) surface properties of the carapace.

4.4.1. Changing habitat

*Tachypleus gigas* is a marine species occurring on sandy beaches and muddy bottoms from the tide-line to depths exceeding 37 m (Mikkelsen 1988). Amplexed pairs migrate from deeper regions to nearshore waters, with the male riding on the female crab for spawning during highest high tide. Migration occurs once every 2 weeks during highest high tide, and then crabs move back to their natural habitat with ebbing tide. Breeding of these crabs is year round (Chatterji 1994). Such a habitat change may exert stress on the diatoms and macro-epibionts. This has been suggested as a problem for epizoans on sea snakes (Key et al. 1995), sea turtles (Caine 1986) and epizoic bryozoans on the horseshoe crab *Carcinoscorpius rotundicauda* (Key et al. 1996). Desiccation and inhospitable depths may cause dislodgement/mortality of epibionts. This investigation revealed the dominance of barnacles and bryozoans in the epibiotic community. *B. amphitrite* is an eurytolerant organism, cosmopolitan in distribution, whose range extends to fringes of the marine environment including the supra-littoral zone. *B. amphitrite* has been reported to
occur to depths of 40 m (Hutchins 1952). Owing to this broad distribution, the physiological stresses caused by changing habitat may be considered negligible. During nesting, crabs spend considerable time on the beach laying eggs. Bryozoans are sessile, colonial animals, commonly encountered in sub-tidal regions (Menon 1972). Menon (1973) also observed that *Membranipora* sp. generally prefer lower levels, i.e. 0.5 to 3 m below tidal levels. This natural distribution range indicates that subaerial exposure can have a negative impact on the epizoic bryozoans. In the case of diatoms, such exposure may not markedly affect the community as several diatom genera are known to tolerate desiccation even at higher temperatures, due to the production of exopolysaccharides which function as antidesiccants (Evans 1959, Hostetter and Hoshaw 1970, Davis 1972, Hoagland et al. 1993). On the other hand, migration of the crabs to deeper waters may curtail the proliferation of diatoms due to light limitations.

4.4.2. Mechanical abrasion and surface availability

During mating, the male, which is almost always smaller, grasps the posterior half of the carapace of the female with the modified pincers of the second pair of feet, thus covering about 70 to 80% of the female opisthosoma. Macro-epibionts were not found in the posterior area of the carapace (i.e. the covered region) of females. The availability of undisturbed surface for macro-epibionts is less on female carapace, while the whole of the male carapace is exposed for epibiont colonization. The amplexed pairs remain in such a position for a considerable time, which further reveals that mating activities have the potential to prevent further epibiosis in the protected region of females. In the case of *Limulus polyphemus*, another species of horseshoe crab seen along the Atlantic and Gulf coasts of North America, amplexed pairs never separate, even after spawning (Barnes 1980). This phenomenon of prolonged amplexus, in which the male remains joined to the female during non-reproductive periods, has been explained as a mechanism to ensure
access to the female by the male at the time of spawning (Rudloe 1980). In the case of turtles, mating activities probably do not markedly affect carapace communities. Both the sexes have similar distributions of epibionts (Caine 1986). Turtles amplex only during breeding season and separate after mating so that both the sexes are exposed wholly for epibiosis.

During nesting, female horseshoe crabs generally bury themselves to the level of the lateral eyes. Females remain in this position, with occasional digging movements, for some time until egg laying and external fertilization occurs (Cohen and Brockman 1983). Once the fertilization is over, the female buries the eggs and begins to excavate the next nest. The friction developed between the sediment and the carapace of the female during nesting can dislodge or cause mortality of diatoms and macro-epibionts on females. Burying behavior of basibionts/hosts adversely affects the settlement and survival of epibionts (Mori and Zunino 1987, Abello et al. 1990, Becker and Wahl 1996). Barnacles were not found on the steep rim surrounding the cardiopthalmic region (Prs1 and Prs2) (Fig. 4.2; Table 4.4). This portion of the carapace is buried in the sediment during the nesting period.

Most shore crabs exhibit grooming activities (cleaning their carapace with the aid of appendages) as a defense mechanism to prevent fouling. Horseshoe crabs do not possess this capability, as their appendages do not extend beyond the edge of the carapace.

4.4.3. Requirements of epizootic larvae

Differences in larval requirements for undergoing metamorphosis, for example phototaxis, surface roughness or chemistry, may influence the distribution of epibionts on the carapace. Most of the bryozoan larvae are known to be negatively phototropic at the time of metamorphosis, while most of the barnacle larvae (cyprids) are positively
phototropic (Thorson 1964). Such differences in larval behavior have been used to interpret the distribution differences of barnacles and bryozoans on the carapace of portunid crabs, e.g. *Bathynectes piperitus* Manning and Holthuis, 1981 (Gili et al. 1993). Mapping studies in this investigation revealed that most of the barnacles were concentrated around entapophyseal pits on the posterior sloping opisthosoma, and on the opercular pleurite adjacent to the prosomal genal angle, along the prosoma longitudinal furrow and over the ophthalmic ridge, suggesting rugophilic (roughness seeking) and rhaeophilic (turbulence seeking) behavior on the part of the cyprid larva (Fig. 4.2; Table 4.4). Bryozoans concentrated closer to movable marginal spines, suggesting rugophilic (groove seeking) behavior. The flanks of the prosoma bore bryozoans, suggesting geophobic (antigravitational) behavior by settling bryozoan larvae (Gore 1995).

4.4.4. Surface properties of the carapace

The properties of the substrata have considerable influence on the metamorphosis of barnacle and bryozoan larvae and are well documented in biofouling studies (Rittschof and Costlow 1989, Maki et al. 1989, 1990, 1994, Anil and Khandeparker 1998). Wettability of a given surface plays an important role in the slime film formation and in attachment of settling larvae (Rittschof and Costlow 1989). The results of the surface wettability measurement indicated the male carapace to be slightly more hydrophobic than the female carapace. The study also revealed that the micro-epibiont population differed on male and female carapaces. Such a difference can also be influenced by the observed variations in the wettability. The electron microscopic evidence revealed that the male carapace is comparatively rougher than the female carapace (Fig. 4.4a). Star-shaped openings were also observed in the female carapace in Prs1 and Prs2 regions, suggesting the opening of pore glands (Fig. 4.4b). Mikkelsen (1988) suggested that horseshoe crabs keep their surfaces clean from ectocommensals and epiphytes by means
of a glycoprotein exudate produced by hypodermal glands and secreted through the carapace. Females harbor large numbers of eggs on the ventral surface of the abdominal appendages. The eggs are toxic. The toxicity of the eggs has been related to the production of tetrodotoxin (Ho et al. 1994a, b). Strong alkaloids, which are toxic, have also been identified in the eggs and tissue of *T. gigas* and *C. rotundicauda* (Mikkelsen 1988). Toxic compounds secreted through the carapace by means of pore canals in females may also play an important role in the control of epibiosis and deserves attention in future efforts. Further evaluation of this aspect would enhance the understanding of epibiosis control in horseshoe crabs and also provide new insights to the development of antifouling technology. In lieu of this male and female horseshoe crab secretions was extracted and its antifouling properties was evaluated. Diatom assays revealed that female horseshoe crab secretions inhibited the growth of *A. coffeaeformis* but the growth was normal with male horseshoe crab secretions and controls (Fig. 4.13). In case of *N. transitans* var. *deresa* f. *delicatula* the growth was normal with both male and female horseshoe crab and controls (Fig. 4.13). Field observations of diatom flora on male and female horseshoe crabs revealed that *Navicula* spp. was the most dominant form among the diatoms encountered in the community (Fig. 4.6). This indicates that the secretions of female horseshoe crabs have the species-specific potential for the control of diatom epibiosis and may be one of the causative agents for the gender differences in the epibiotic community structure.