

## 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).

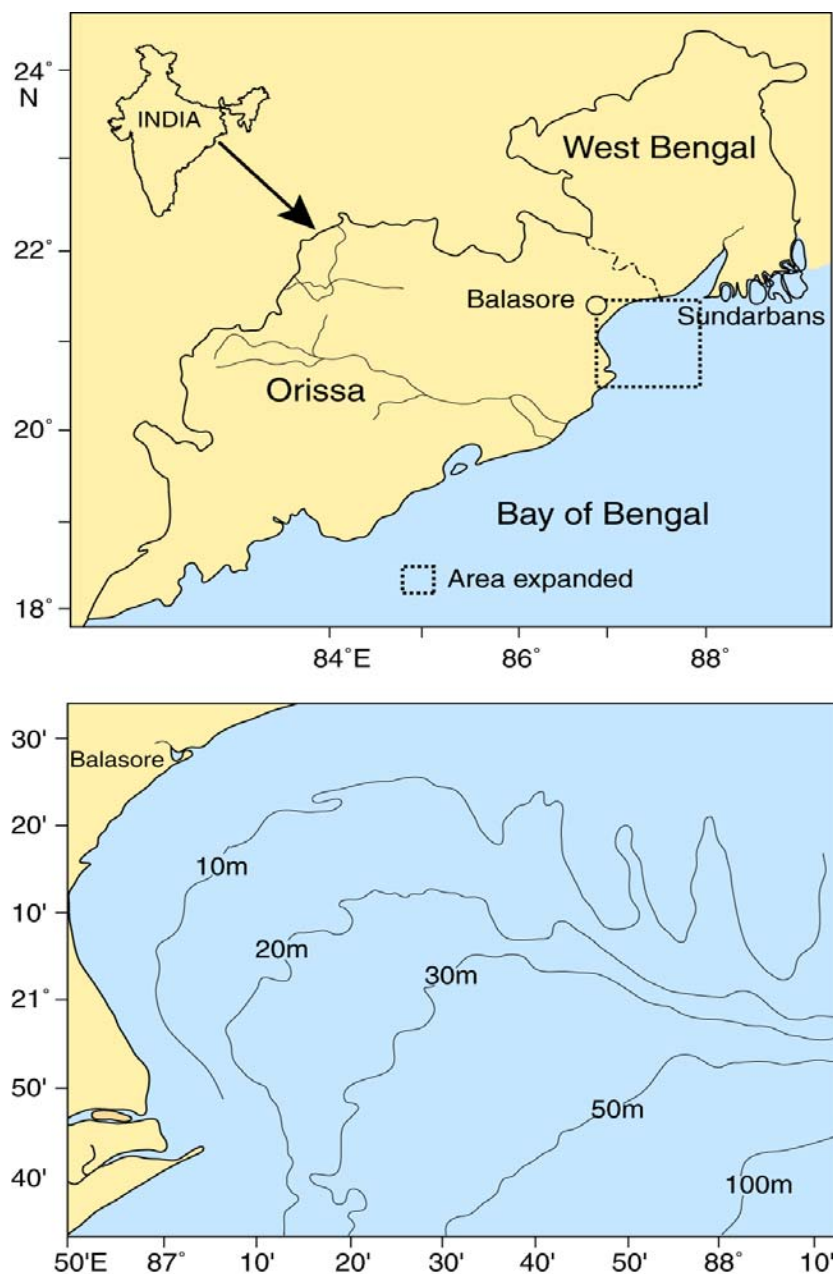
Horseshoe crabs act as moving substrata for simple to complex communities of small marine organisms. Horseshoe crabs carry a variety of epibionts on their external surface, including green algae, diatoms, coelenterates, flatworms, mussels, oysters, annelids, barnacles, tunicates, bryozoans, isopods, 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 anecdyasis 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 anecydysis. 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



**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.

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

determined by the drop-spread method as described by Gerhart et al. (1992), using HPLC-grade water and methanol. The spread of 25  $\mu\text{l}$  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 dropspread 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).

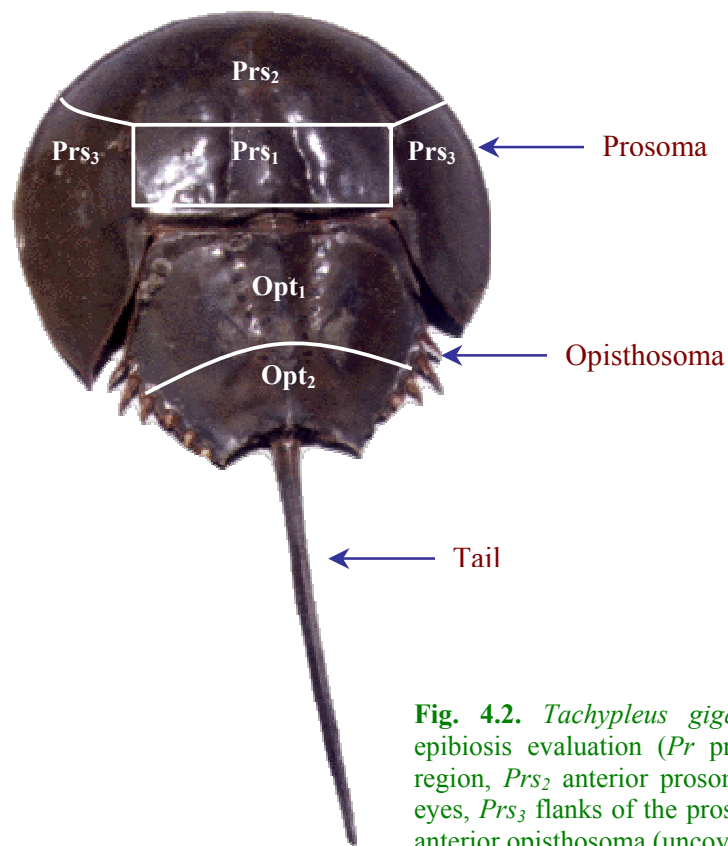
#### *4.2.1. Enumeration of diatoms*

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 ( $\text{cells dm}^{-2}$ ) 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).



**Fig. 4.2.** *Tachypleus gigas* Carapace demarked for epibiosis evaluation (*Pr* prosoma: *Prs<sub>1</sub>* cardiophthalmic region, *Prs<sub>2</sub>* anterior prosoma up to the level of lateral eyes, *Prs<sub>3</sub>* flanks of the prosoma; *Opt* opisthosoma: *Opt<sub>1</sub>* 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 (*Prs<sub>1</sub>*, *Prs<sub>2</sub>*, *Prs<sub>3</sub>*, *Opt<sub>1</sub>* and *Opt<sub>2</sub>*) 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 ( $29 \pm 2$  °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 ( $15^{\circ} 27'$  N and  $73^{\circ} 48'$  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  $20 \pm 1$  °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  $\text{mm}^{-2}$ . In each of these



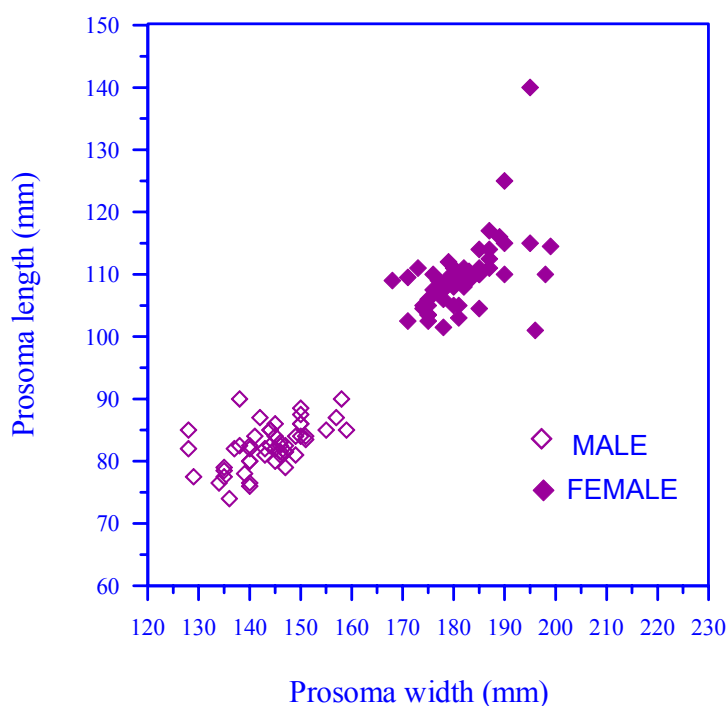
assay conditions, enumeration of the diatom numbers was carried out at an interval of 24, 48 and 72 h.

#### 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<sub>2</sub>SO<sub>4</sub> + 18 ml ethyl alcohol) and heated at 110<sup>0</sup> 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*. Prosoma size of male and female horseshoe crabs used in the study.

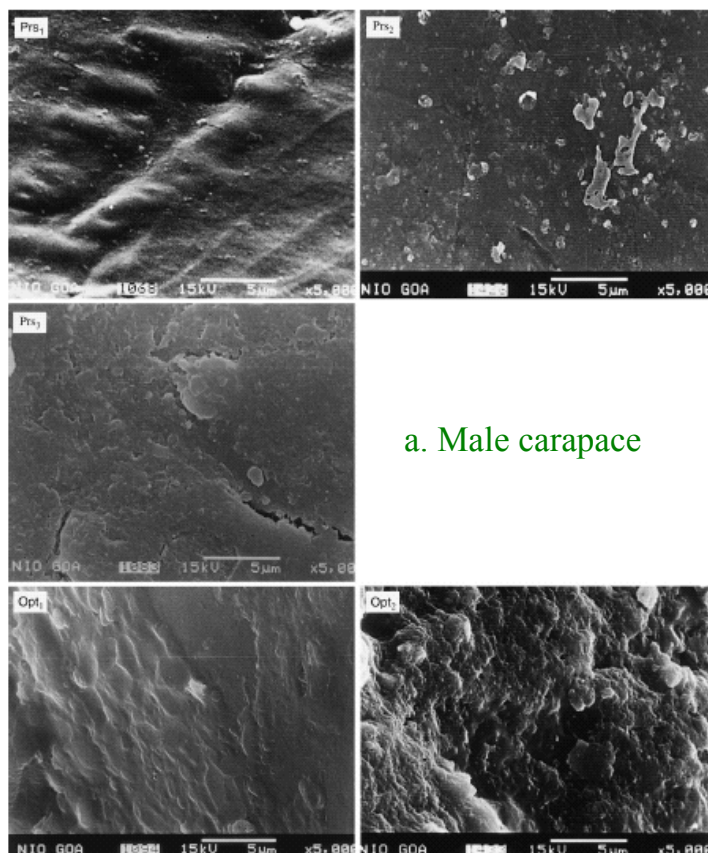
The surface area of the prosoma was approximately 75% greater than the opisthosoma in both sexes. The surface area of the female's prosoma and opisthosoma was 55 to 65% greater than that of the males.

#### *4.3.1. Surface properties of the carapace*

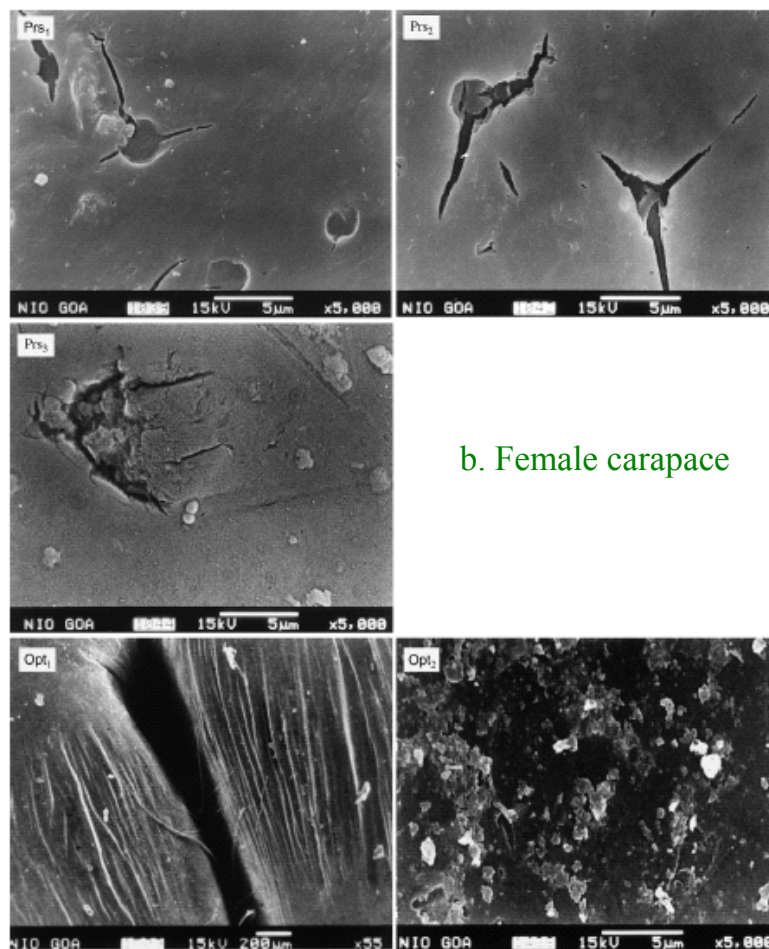
The whole body is covered by tough chitinous exoskeleton, which is sage green in color. The males were lighter in color than the females. The results obtained for surface wettability indicated the male carapace to be slightly more hydrophobic ( $\text{SHM} = 14.5 \pm 2.34$ ) than the female carapace ( $\text{SHM} = 18.7 \pm 1.8$ ). 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



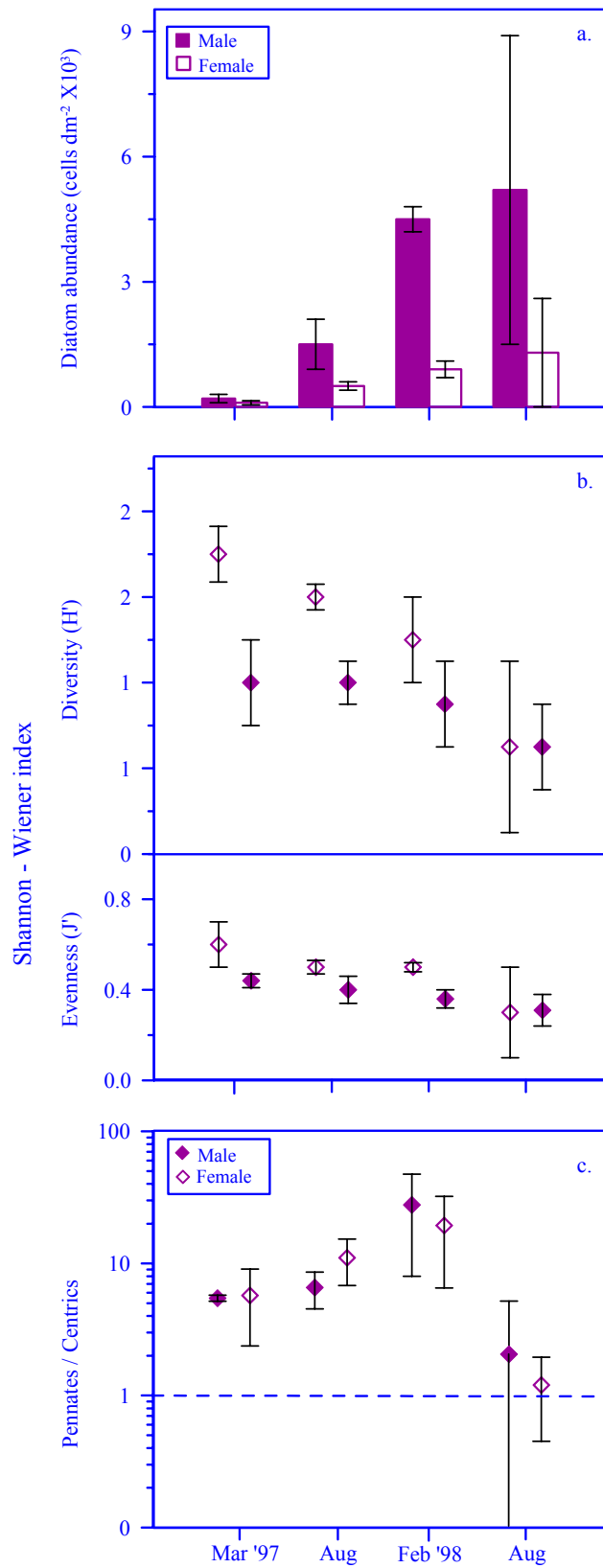
rough as compared to the smooth carapace of females. Star-shaped openings were observed on the female carapace in the cardiophthalmic region and posterior part of the rim surrounding the cardiophthalmic (posterior prosoma) region (Fig. 4.4b, Prs1 and Prs2). Such openings were not seen on any of the male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



**Fig. 4.4.** *Tachypleus gigas*. Scanning electron micrographs of horseshoe crab carapaces. **a** Male carapace: surface texture is rough in Prs<sub>1</sub>, Opt<sub>1</sub> and Opt<sub>2</sub> regions. **b** Female carapace : surface texture is comparatively smooth. Star-shaped openings were seen in Prs<sub>1</sub>, and Prs<sub>2</sub> regions. Location of Prs<sub>1</sub>, Prs<sub>2</sub>, Prs<sub>3</sub>, Opt<sub>1</sub> and Opt<sub>2</sub> on the carapace is as in Fig. 4.2.

#### 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<sup>-2</sup>), generic diversity (H') and evenness values (J') were lower for females than for males (Figs. 4.5a, b).



**Fig. 4.5.** *Tachypleus gigas*. A comparison of **a** diatom cell abundance (cells  $\text{dm}^{-2} \times 10^3$ ), **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<sup>-2</sup>) 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).

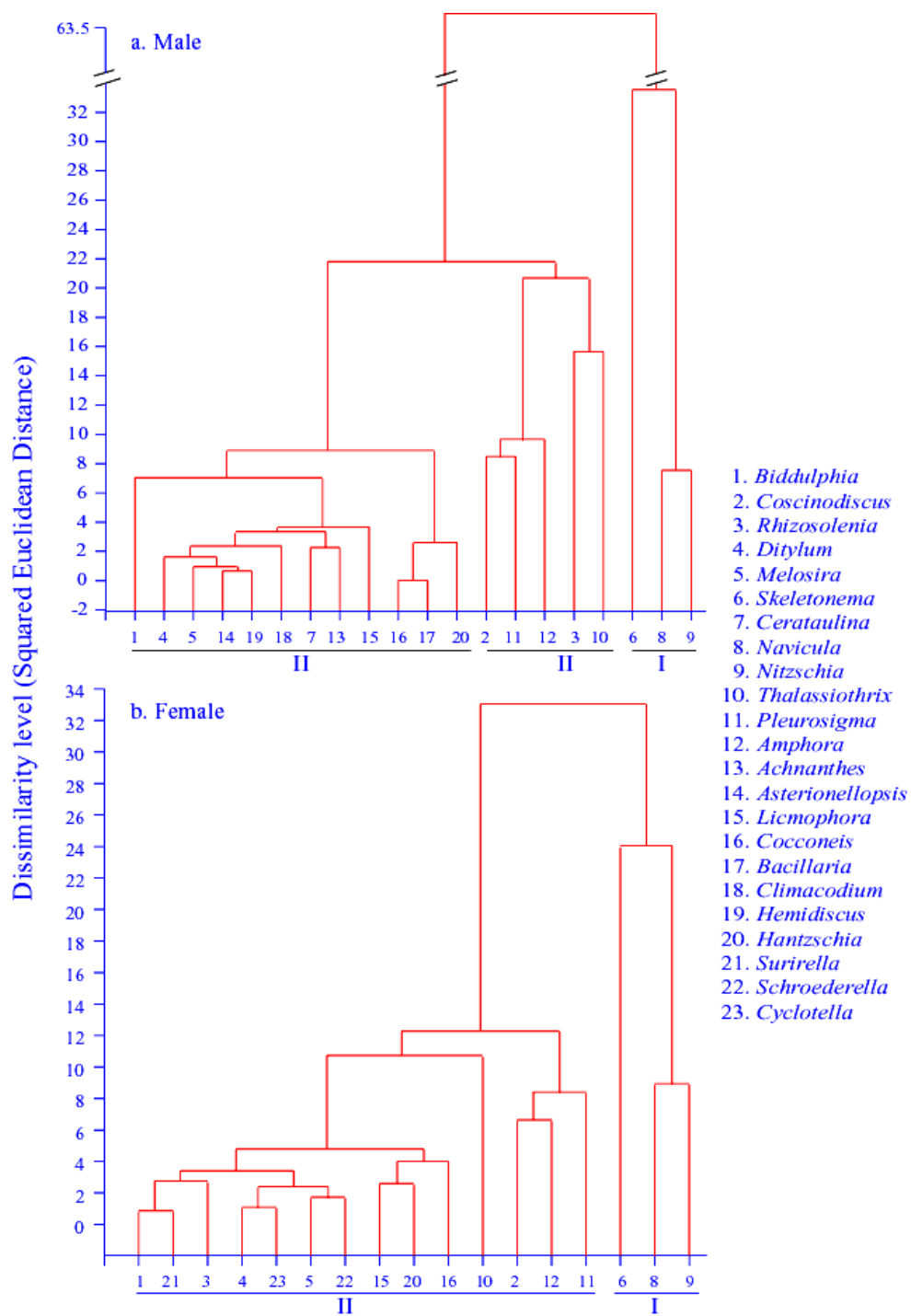
**Table 4.1.** *Tachypleus gigas*. Results of two-way ANOVA with uneven sample sizes comparing diatom density (cells dm<sup>-2</sup>) between sexes (male and female) over period of sampling.

	<i>df</i>	SS	MS	<i>F<sub>s</sub></i>	<i>p</i>
Subgroups (8: 4 samples for each sex)	7	5.9	0.8	6.5	≤ 0.005
Sampling period (4)	3	4.1	1.4	10.4	≤ 0.001
Sexes	1	1.6	1.6	12.4	≤ 0.005
Sampling period X sexes	3	0.2	0.1	0.6	
Error	14	1.8	0.1		
Total	21	7.7			

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).

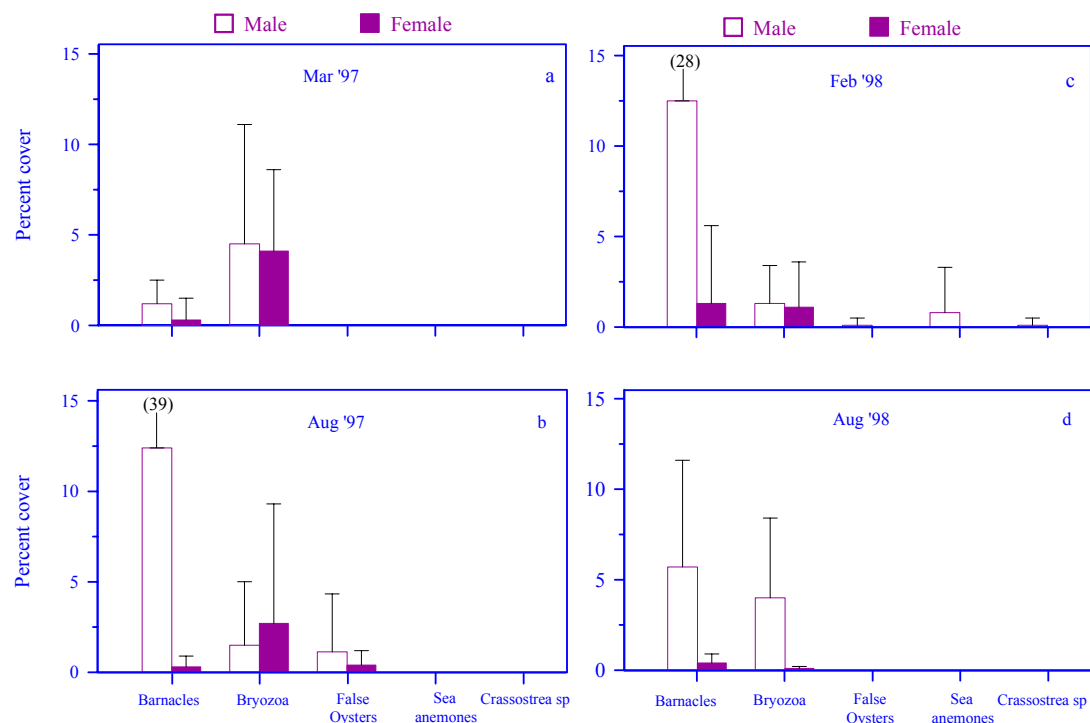
#### 4.3.4. Macro-epibionts

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



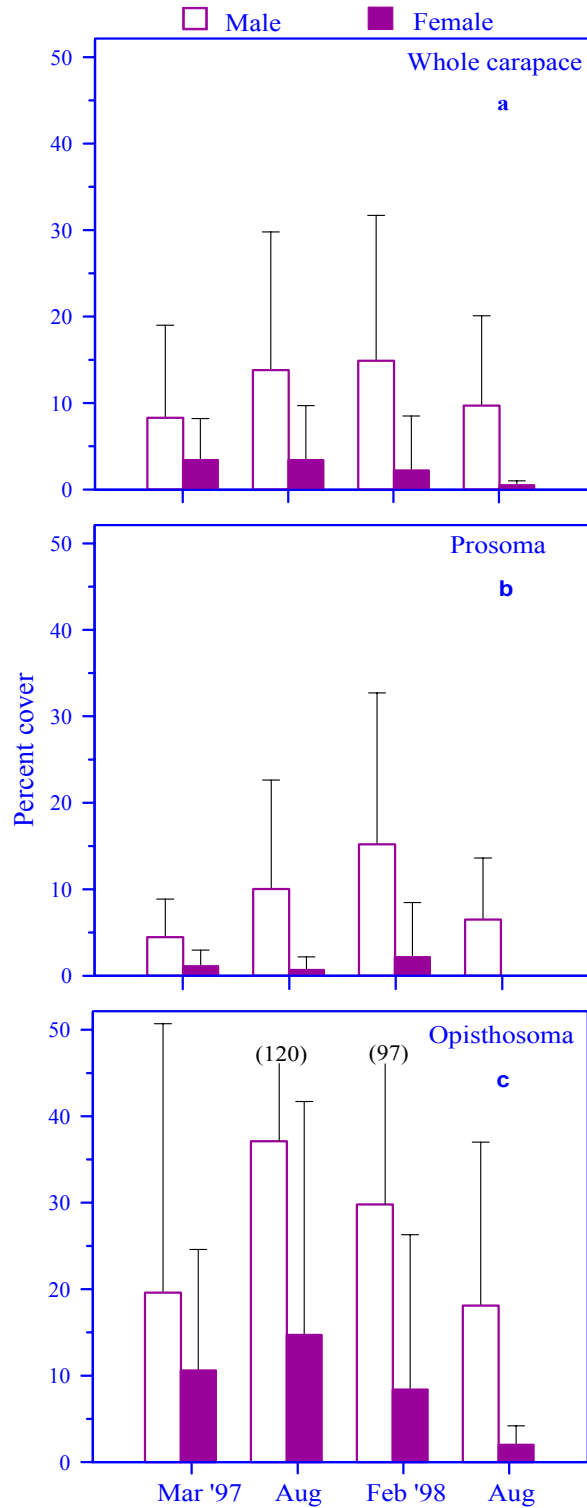
**Fig. 4.6.** *Tachypleus gigas*. Generic cluster dendrograms of the epibiotic diatom community from **a.** male and **b.** female horseshoe crab.

sampling. Acorn barnacles (*B. amphitrite*) and encrusting bryozoans (*Membranipora* sp.) were the most abundant forms encountered in terms of coverage (Fig. 4.7).



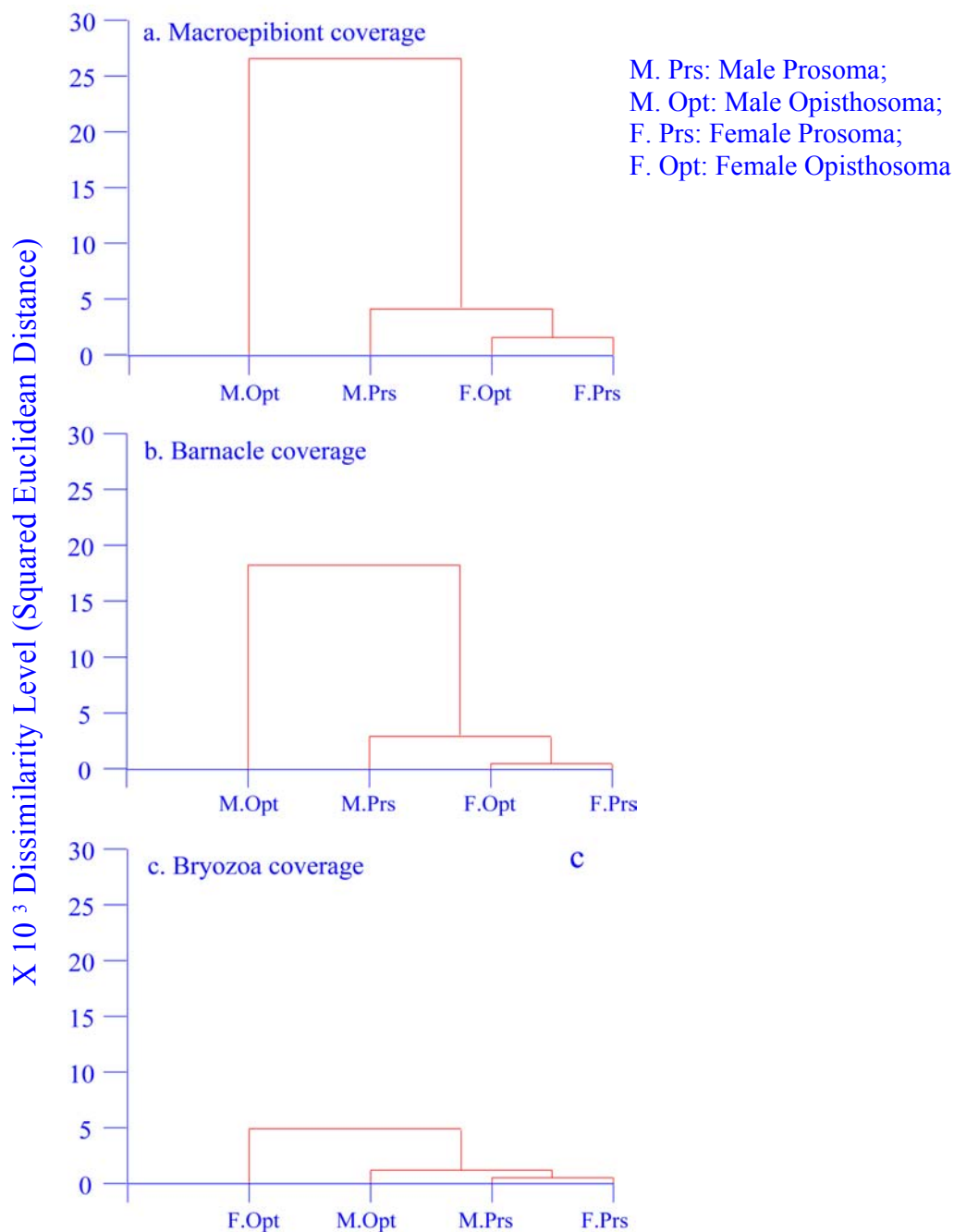
**Fig. 4.7.** *Tachypleus gigas*. Comparison of coverage of macro-epibionts on male and female horseshoe crab carapaces during different sampling periods (a to d). Error bars indicate standard deviation.

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.** *Tachypleus gigas*. Dendrograms 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).

	<i>df</i>	SS	MS	$F_s$	<i>p</i>
Parts (Prs & Opt)	1	189.9	189.9	14.6	≤ 0.001
Sexes	1	3555.4	3555.4	27.4	≤ 0.001
Parts X Sexes	1	712.4	712.4	5.5	≤ 0.050
Error	180	23394.4	129.9		
Total	183	27852.1			

Barnacle abundance (ind dm<sup>-2</sup>) 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

**Table 4.3.** *Tachypleus gigas*. Results of two-way ANOVA comparing barnacle (*Balanus amphitrite*) coverage between sexes (male and female) and between different parts (prosoma and opisthosoma) of carapace (*Prs* Prosoma; *Opt* Opisthosoma).

	<i>df</i>	SS	MS	$F_s$	<i>p</i>
Parts (Prs & Opt)	1	772.7	772.7	8.3	≤ 0.005
Sexes	1	2477.5	2477.5	26.5	≤ 0.001
Parts X Sexes	1	525.1	525.1	5.6	≤ 0.025
Error	180	16806.9	93.4		
Total	183	20582.2			

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 cardiophthalmic region (i.e. the anterior

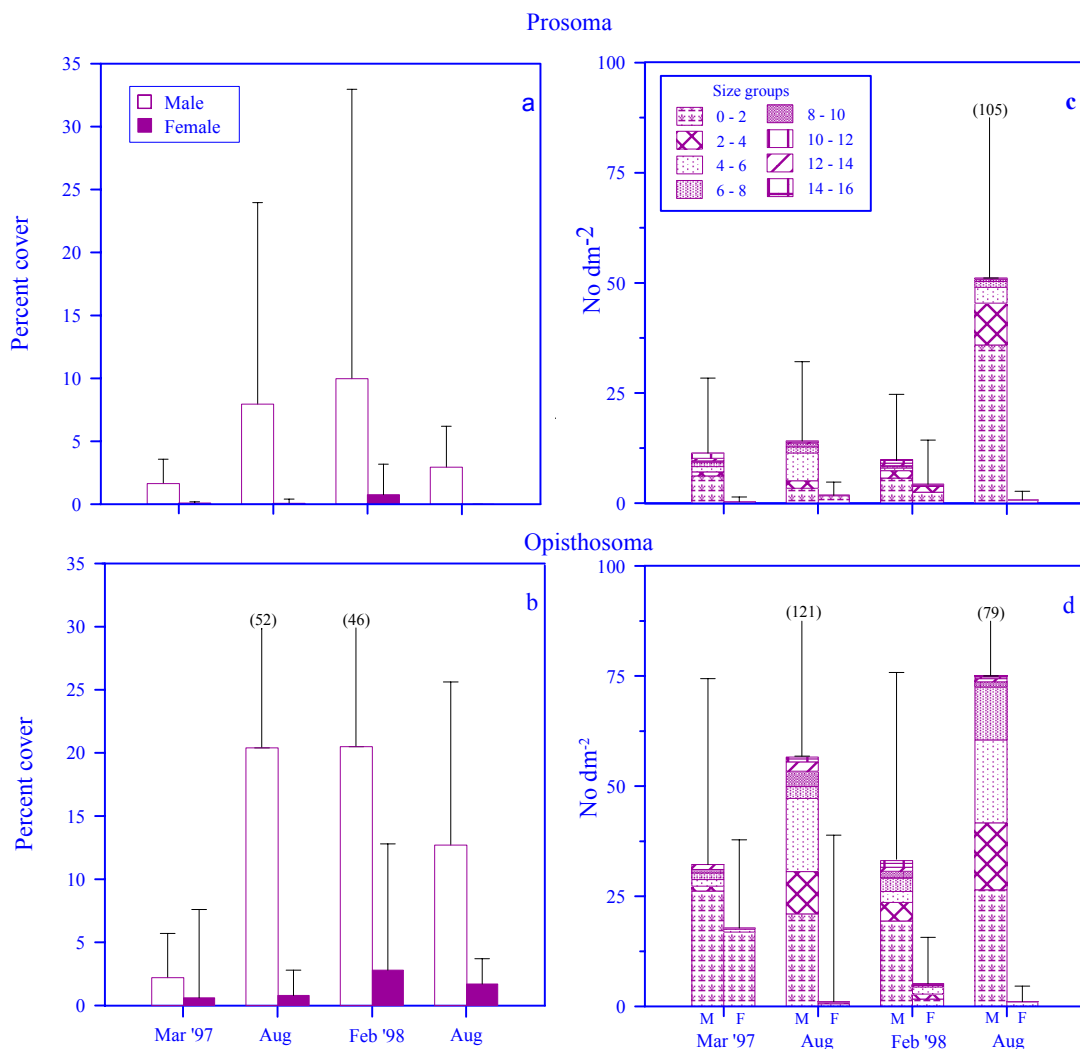
portion of the prosoma which is subjected to mechanical abrasions) and in the posterior area of the opisthosoma (i.e. the area covered by males) (Table 4.4). The abundance of

**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; *Prs*<sub>1</sub> cardiopthalmic region; *Prs*<sub>2</sub> anterior prosoma up to the level of lateral eyes; *Prs*<sub>3</sub> flanks of the prosoma; *Opt*<sub>1</sub> anterior opisthosoma (uncovered region); *Opt*<sub>2</sub> posterior region (covered region)).

	Prs <sub>1</sub>		Prs <sub>2</sub>		Prs <sub>3</sub>		Opt <sub>1</sub>		Opt <sub>2</sub>	
	M	F	M	F	M	F	M	F	M	F
Barnacle spat	12 ± 58	2 ± 58	1 ± 5	0.3 ± 2	0.2 ± 1	0.2 ± 1	7 ± 16	4 ± 8	2 ± 8	1 ± 7
Adult	6 ± 11	1 ± 2	4 ± 8	0.1 ± 1	1 ± 3	0.1 ± 2	7 ± 15	2 ± 7	5 ± 12	0.2 ± 13
Bryozoa (%)	15	2	15	2	24	14	33	30	28	16

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).

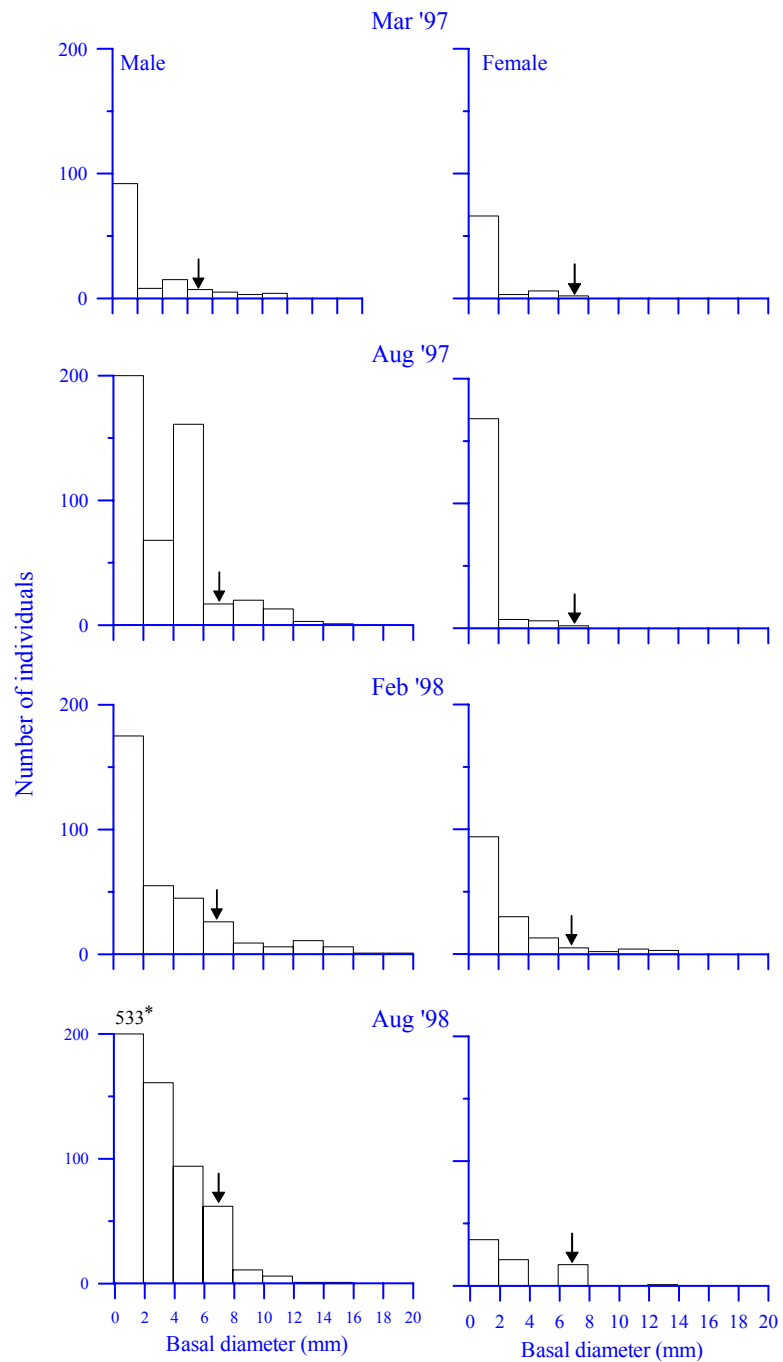
Encrusting bryozoans were the other dominant organisms contributing to total macro-epibiont coverage (Fig. 4.7). The bryozoan coverage was greater in the case of males than females (Fig. 4.7). Encrusting bryozoan coverage was found to be less on the prosoma than on the opisthosoma in both sexes (Fig. 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).



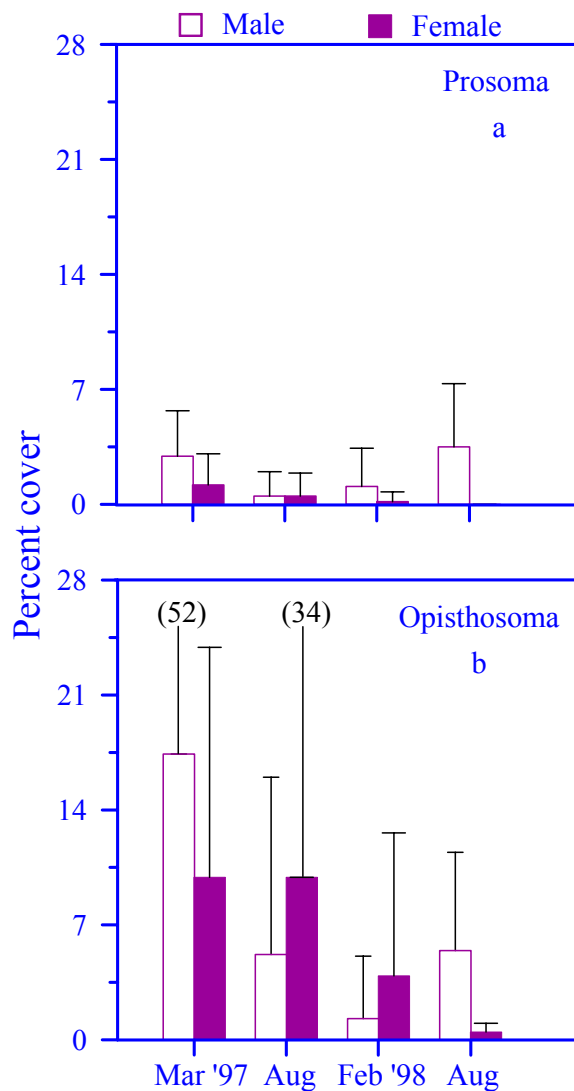
**Fig. 4.10.** *Tachypleus gigas*. Comparison of *Balanus amphitrite* **a, b** % cover and **c, d** abundance (ind dm<sup>-2</sup>, of different size groups) between male and female horseshoe crabs on **a, c** prosoma and **b, d** opisthosoma during different sampling periods. Error bars indicate standard 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).

	<i>df</i>	SS	MS	<i>F<sub>s</sub></i>	<i>p</i>
Parts ( <i>Prs</i> & <i>Opt</i> )	1	234.9	234.9	6.60	≤ 0.01
Sexes	1	0.5	0.5	0.01	ns
Parts X Sexes	1	39.4	39.4	1.10	ns
Error	180	6441.5	35.8		
Total	183	6716.3			



**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).



**Fig. 4.12.** *Tachypleus gigas*. Comparison of bryozoan coverage (*Membranipora* sp) between male and female horseshoe crabs on **a.** prosoma and **b.** opisthosoma during different sampling periods. Error bars indicate standard deviation.

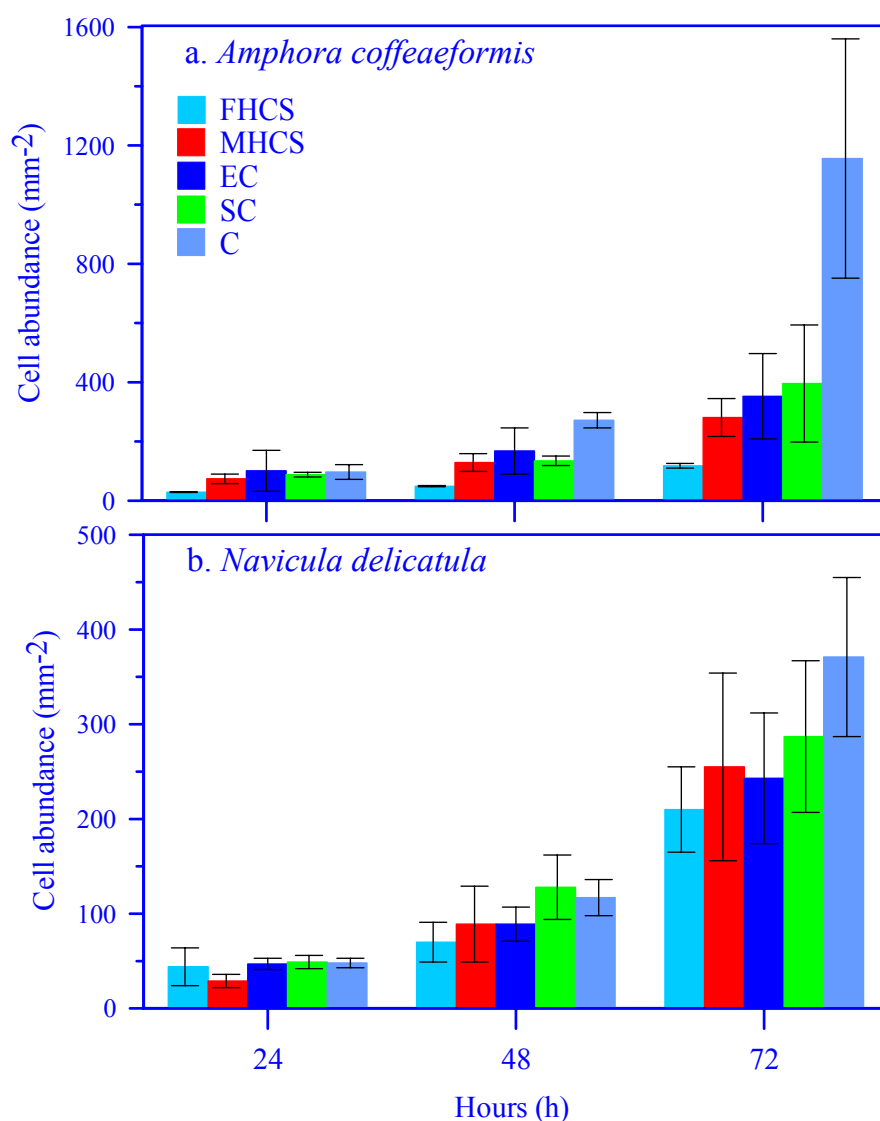
#### 4.3.5. Diatom assay

##### 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. 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  $\text{mm}^{-2}$ ), male secretion (29 to 255 cells  $\text{mm}^{-2}$ ), experimental control (47 to 243 cells  $\text{mm}^{-2}$ ), methanol control (287 to 949 cells  $\text{mm}^{-2}$ ) and sea water control (48 to 371 cells  $\text{mm}^{-2}$ ) (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: Exnerimental 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 4.6.** *Tachypleus gigas*. RF values for secretions of female horseshoe crab (FHC) and male horseshoe crab (MHC) and experimental control (EC).

Sl. No	FHC	MHC	EP
1	0.193	0.204	0.182
2	0.636	0.636	0.636
3	0.761	-	-



**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 anecdyosis; 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 a 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. *derasa* 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.

## Chapter 5

Influence of diatoms on cyprid metamorphosis of *Balanus amphitrite*

## 5.1. INTRODUCTION

Most of the sessile invertebrates produce pelagic larva as part of their life history. The final stage of the larvae plays a crucial part in their life cycle, as its sole purpose is to search and attach to the most suitable site on the substratum for further development into sedentary juvenile individuals. The commencement of larval settlement and metamorphic processes is possibly triggered by environmental (physical and biological) stimuli. The onset of larval surface exploration and substratum choice for attachment is influenced by the biofilm on the surfaces (Zobell and Allen 1935). Several studies suggest that biofilms can play an important role in mediating settlement and metamorphosis of invertebrate larvae (Kirchman and Mitchell 1981, 1983; Kirchman et al. 1982a, 1982b, Maki and Mitchell 1985, 1986; Maki et al. 1988, 1989, 1990, 1992, 1994; Mitchell 1984, Mitchell and Kirchman 1984; Mitchell and Maki 1988, Rittschof et al. 1992, Rodriguez et al. 1995, Zhao and Qian 2002). Biofilm is a complex network of microorganisms (bacteria, diatoms, protozoa and fungi) and their extracellular polymeric substances. Amongst these, diatoms are the earliest eukaryotic colonizers of submerged surfaces and one of the most conspicuous organisms in biofilms. Attachment of diatoms is a more complicated process than bacteria (Cooksey and Cooksey 1995) and is invariably associated with the production of extracellular polysaccharides in the form of pads, envelopes, stalks or tubes (Chamberlain 1976, Daniel et al. 1987, Hoagland et al. 1993). Exopolysaccharides appear to play many functions in the adherent biofilms including motility, protection and maintenance (Characklis and Cooksey 1983, Costerton et al. 1987, Hoagland et al. 1993). These exopolymers are composed of polysaccharides with variable amount of proteins. These extracellular polymeric substances either contribute to the biofilm matrix (Cooksey 1992, Costerton et al. 1994) or are released by



the microorganisms to the surrounding medium as free EPS (Sutherland 1985, Beech 1990, Beech et al. 2000, Khandeparker and Bhosle 2001).

Biofilm formation on a substratum modifies its surface chemistry (Characklis and Cooksey 1983) and strongly influences the larval recruitment that leads to economic problems in shipping industry and industrial aquatic processes. Marine invertebrate larvae are presented with a wide range of cues as they approach a substratum. These cues may be physical ones or biologically derived chemical cues associated with bacteria, microflora and microfauna. Microbial biofilms have generally been examined as a stimulus for the settlement of macrofouling organisms (Crisp 1974). The larvae may use specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions at a site. Numerous relevant literature report that biofilms can have varying effects on larval attachment (Kirchman et al. 1982a, Weiner et al. 1985, 1989, Maki et al. 1988, 1989, 1990, 1992, Szewzyk et al. 1991, Holmstrom et al. 1992, Pawlik 1992, Avelin Mary et al. 1993, Rodriguez et al. 1993, Todd and Keough 1994, O' Connor and Richardson 1996, Wieczorek and Todd 1998, Anil and Khandeparker 1998, Qian 1999, Khandeparker et al. 2002). In these studies, the inductive effect of natural biofilms on larval settlement is attributed to bacteria and their components in biofilms. Recently a thraustochytrid protist, detected in marine microbial films has been shown to induce settlement of the barnacle *Balanus amphitrite* (Raghukumar et al. 2000). Surprisingly till date, elucidation of invertebrate larval settlement cues from the diatoms and their components is limited although their proportion in biofilms is high, especially in illuminated areas.

Earlier studies on larval induction of marine invertebrates by benthic diatoms have mainly focused on invertebrate species with grazing juveniles, such as abalones (Ebert

and Houk 1984, Kawamura and Kikuchi 1992, Slattery 1992, Bryan and Qian 1998, Daume et al. 1999), Sea urchins (Tani and Ito 1979) and Sea cucumbers (Ito and Kitamura 1997). Until recently, very few studies have been focused on the larval settlement induction by benthic diatoms with respect to larval settlement behavior of sedentary organisms such as barnacle, *Balanus balanoides* (Le Tourneux and Bourget 1988) and polychaete, *Hydroides elegans* (Harder et al. 2002). These studies mainly focused on the influence of biofilms dominated by benthic diatoms. In the present study, efforts were made to investigate the influence of diatom films of different densities and their extracellular components on the larval metamorphosis of the barnacle, *Balanus amphitrite*.

*Balanus amphitrite* is a dominant fouling organism found all over the world and is the major target organism in biofouling studies as well as in the development of antifouling technology. *B. amphitrite*, an acorn barnacle is an important model organism for these studies because of its rapid larval development, the ease of raising synchronous mass cultures and the predictable settlement in static conditions. . This barnacle species is eurytolerant (Crisp and Costlow 1963, Iwaki 1981, Anil 1991, Anil et al. 1995), breeds throughout the year (Karande 1967; Anil 1986) and is a dominant fouling organism in the fouling community in Indian waters (Karande 1967; Purushotham and Satyanarayan Rao 1971; Anil 1986; Venugopalan and Wagh 1990; Fernando 1990; Satyanarayan Rao and Balaji 1988; Rao 1989; Santhakumaran 1989). It has been reported from both, east and west coasts of India (Karande 1967,1974; Fernando 1978; Anil 1986; Pillai 1958). The capabilities of five diatom species, belonging to different genera viz *Navicula* and *Amphora* dominant in microfouling and benthic microalgal community, in inducing larval metamorphosis of *Balanus amphitrite* was explored. The diatom species isolated

from natural biofilms were used as candidate organisms. The effectiveness of different settlement inducing components from these diatoms was assessed.

## 5.2. MATERIALS AND METHODS

### 5.2.1. *Culturing conditions and source of algal material*

Five species of pennate diatoms belonging to two genera *Amphora* (*A. coffeaeformis* and *A. rostrata*) and *Navicula* (*N. crucicula*, *N. transitans* var. *derasa* f. *delicatula* and *N. subinflata*) were used in this study. The cultures are maintained in Marine Corrosion and Materials Research Division, National Institute of Oceanography, Goa, India. *A. rostrata*, *N. crucicula* and *N. subinflata* were originally isolated from the biofilm, whereas *A. coffeaeformis* and *N. transitans* var. *derasa* f. *delicatula* were isolated from the intertidal sediments of Dona Paula Bay, west coast of India (15° 27' N and 73° 48'E). Cultures of these species were maintained in the exponential growth phase by weekly reinoculation into fresh culture medium, prepared in autoclaved seawater (~ 35 psu) enriched with nutrients (f/2 media; Guillard and Ryther 1962). Cells were grown in Erlenmeyer flasks (100 ml) containing 50 ml culture medium and were maintained at 27 ±1°C under a light regime of 12 h light: 12 h dark photocycle.

### 5.2.2. *Extraction of diatom exopolymers (EPM)*

Prior to the extraction of diatom exopolymers, diatom cultures were made bacteria free in order to avoid the contribution of bacterial exopolymers. A two-step method (including physical and chemical methods) was employed to obtain the bacteria free culture. Firstly, the diatom cells were given repeated sterile wash (10 times) before and after mild sonication to remove loosely adhered bacterial cells. After this treatment, the diatom cells were incubated overnight in f/2 media containing 1 mg ml<sup>-1</sup> lysozyme to remove gram-negative bacteria (Kim et al. 1999). Subsequent to the lysozyme treatment,

the cultures were again given repeated sterile washings and were checked microscopically for any cell wall permeabilization. The cultures were later incubated in fresh sterile f/2 medium containing antibiotic mixture (1 mg ml<sup>-1</sup> of Penicillin G, 0.5 mg ml<sup>-1</sup> of Streptomycin, and 0.2 mg ml<sup>-1</sup> of Chloramphenicol) for 24 hours. The culture was then transferred to antibiotic free medium and cultivated as above. Confirmation of bacteria free culture was done by spread plate method using Zobell marine agar and by fluorochrome staining by using 4',6-diamidino-2-phenylindole (DAPI). This method of obtaining bacteria free cultures was employed for all the diatom species. These bacteria free cultures were further used for exopolymer extraction and carrying out larval assays.

Mass cultures (4.5 L) of each species were raised in Schott Duran Bottle using f/2 medium, as described above. After attaining stationary phase (16 d), the supernatant was decanted to separate the cells attached to the culture flask. The decanted supernatant was used to isolate planktonic or free EPM and the attached cells were used for the isolation of biofilm EPM.

The biofilm EPM from the attached cells was extracted following the method described by Beech et al. (2000). The attached diatom cells were treated with isotonic Tris buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA) containing 2.5% NaCl and incubated overnight at 4 °C. After incubation, the extracted biofilm EPM and supernatant were centrifuged separately at 6,000 rpm (12,000 g) at 4 °C for 30 minutes followed by a 0.2 µm (Nucleopore) filtration to remove any cell material. The filtrate was concentrated in a stirred ultra centrifugation cell (Amicon, Danvers MA) using an ultra filter with a MW cut off of 1,000D. The carbohydrate content of the filtrate was quantified by using phenol-sulfuric acid method (Dubois et al. 1956), with glucose as standard. At the time of harvesting EPM, cell counts were also made in order to estimate EPM production per

cell. The same procedure was followed for all the diatom species. Isolation of EPM from non-axenic culture of *N. crucicula* and *N. subinflata*, which were abundant in the fouling community, was also carried out by following the similar method, to compare the results of axenic and non axenic conditions. The EPM produced was further used for larval assays and FTIR analysis.

Growth rate and generation time for all the diatom species used in the study were also calculated from the initial count of the inocula and final counts, which were made at the time of harvesting EPM.

### ***5.2.3. Preparation of the adult extract (AE)***

Cyprid larva is discriminating in its choice of settlement site (Knight-Jones 1953; Crisp and Meadows 1962, 1963). Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics. This gregarious feature has been related to settlement pheromone, a glycoprotein present in the adults, referred as arthropodin (Knight-Jones and Crisp 1953, Knight-Jones 1953, Crisp and Meadows 1963). So, an extract was prepared from the adult *B. amphitrite* and it was considered as a positive control in the larval assays.

Adult extract was prepared by following the method described earlier by Larman et. al. (1982). Adults of *B. amphitrite*, collected from the intertidal area of Dona Paula Bay (15° 27' N, 73° 48' E), were brought to the laboratory and cleaned by brushing off the epibiotic growth on their shells using a nylon brush. The animals were then washed and 100-g wet weight of whole adults was crushed with a mortar and pestle using 100 ml of deionised water (RO pure). The supernatant of the crushed mixture was decanted, centrifuged at 12,000 X g for 5 min and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged at 12,000 X g for 5 min and then frozen at -20° C

until further use. The protein content of the extract was estimated following the method described by Lowry et. al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of  $50 \mu\text{g ml}^{-1}$  of AE was used for all assays.

#### **5.2.4. Rearing of *B. amphitrite* larvae**

The life cycle of *B. amphitrite* includes planktotrophic larval development consisting of six naupliar instars and a non-feeding cyprid instar. The first instar nauplii do not feed and molt into the second instar within 1-2 hours. Instars II to VI are phytoplanktotrophic. Nauplii were mass reared in 2-liter glass beakers using filtered seawater of 35 psu on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of  $2 \times 10^5$  cells  $\text{ml}^{-1}$ . The food organism was replenished every day while changing the water. After 5-6 days, the cyprids obtained were siphoned out and stored at  $5^\circ \text{C}$  prior to settlement assays. Two-day-old cyprids were used to carry out the assays. These methods have been described in detail by Rittschof et al. (1984).

#### **5.2.5. FTIR spectroscopy**

Samples were prepared by grinding EPM with potassium bromide, and pressing the mixture to form a pellet. A Fourier transformed infrared spectroscopy (FTIR - model) was used to read absorbances from  $500$  to  $4000 \text{ cm}^{-1}$ . FTIR was done to detect the major structural groups of biofilm and free EPM produced by diatoms.

#### **5.2.6. Assay protocol for Experiments 1 and 2**

Two different experiments were carried out; the schematic representation of which is given in Fig. 5.1. In Experiment 1, the effectiveness of axenic and non-axenic diatom exopolymers, both planktonic and biofilm was investigated. In Experiment 2, the influences of diatom films (both axenic and non-axenic) at different cell densities were

assessed. Along with these experiments, the effect of positive control (AE) on larval metamorphosis was also investigated.

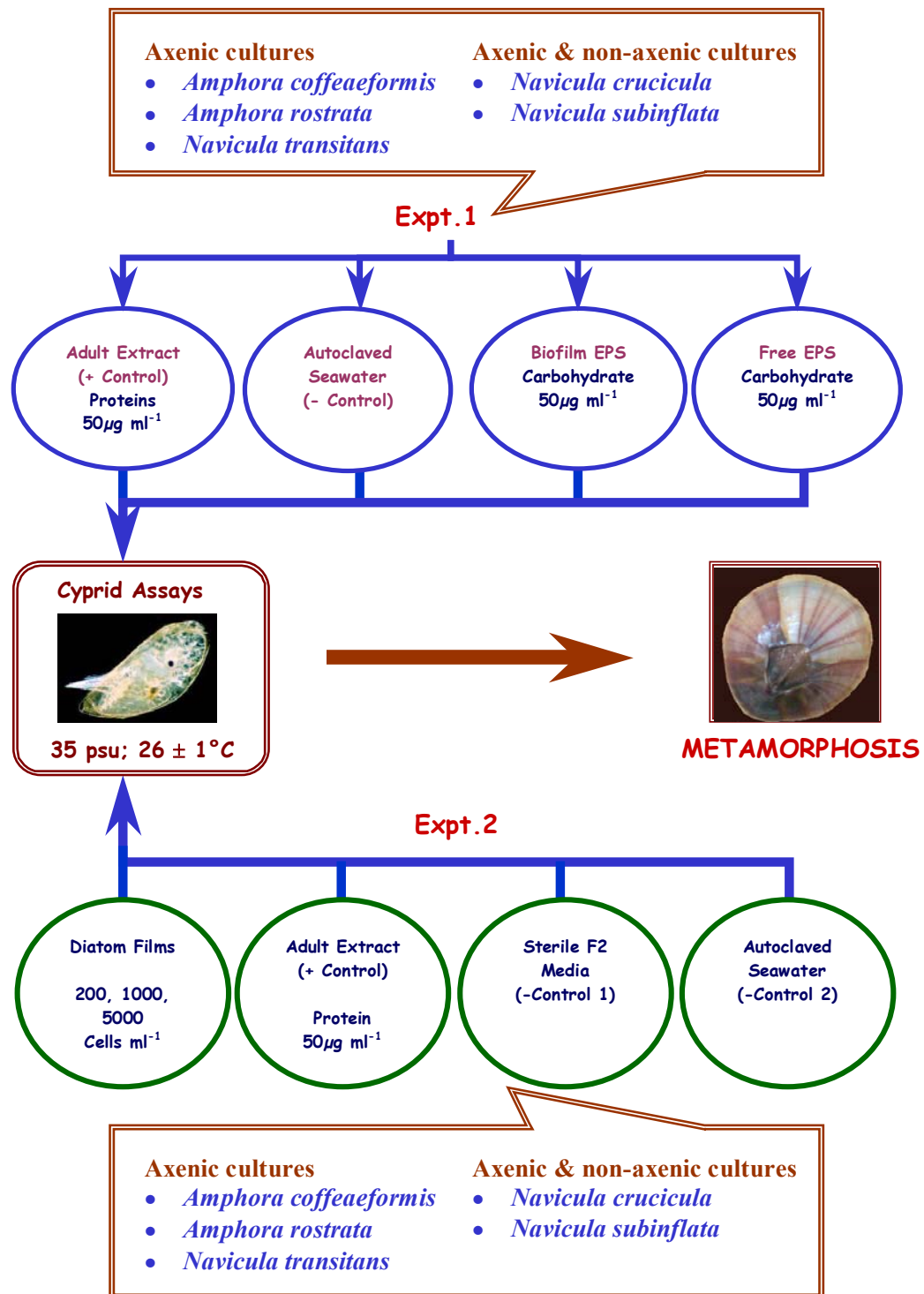


Fig. 1. Schematic representation of the experimental set-up.

### ***5.2.6.a Experiment 1:***

The settlement assays were carried out using 24 well multiwells (Corning -25820). The multiwells were inoculated with biofilm/free exopolymers from axenic/non-axenic diatom cultures. Controls were sterile filtered seawater (FSW) and f/2 medium (as negative controls) and AE (positive control). The inoculated multiwells were rinsed off after three hours by repeated rinsing with autoclaved filtered seawater under a laminar chamber. Subsequently, 1 cyprid was introduced with 2 ml of autoclaved filtered seawater (35 psu). The settlement assays were monitored for a period of four days with an intermittent observation everyday. Assay wells were maintained at  $26 \pm 1^\circ \text{C}$  (12h light: 12h dark photoperiod). The assays were conducted separately with axenic and non-axenic diatoms and were repeated thrice by taking different batches of larvae i.e. in triplicates ( $n \approx 10$  in each of the triplicate).

### ***5.2.6.b. Experiment 2:***

The settlement assays were carried out using 24 well multiwells (Corning -25820). The multiwells were inoculated aseptically under a laminar flow with axenic and non-axenic diatom cultures at densities of 200, 1000 and 5000 cells  $\text{ml}^{-1}$ . After inoculation, the multiwells were kept overnight for the formation of films. The transfer of larvae, monitoring of settlement assays and maintenance of assay wells was the same as described for experiment 1. As in experiment 1, the assays were conducted separately with axenic and non-axenic diatoms. The growth of diatoms in the multiwells was also assessed along with the monitoring of settlement assays.

### ***5.2.7. Data analysis***

The data obtained are described as percentages of metamorphosed larvae in each assay condition. The data on metamorphosis (%) was arcsine transformed to ensure normality

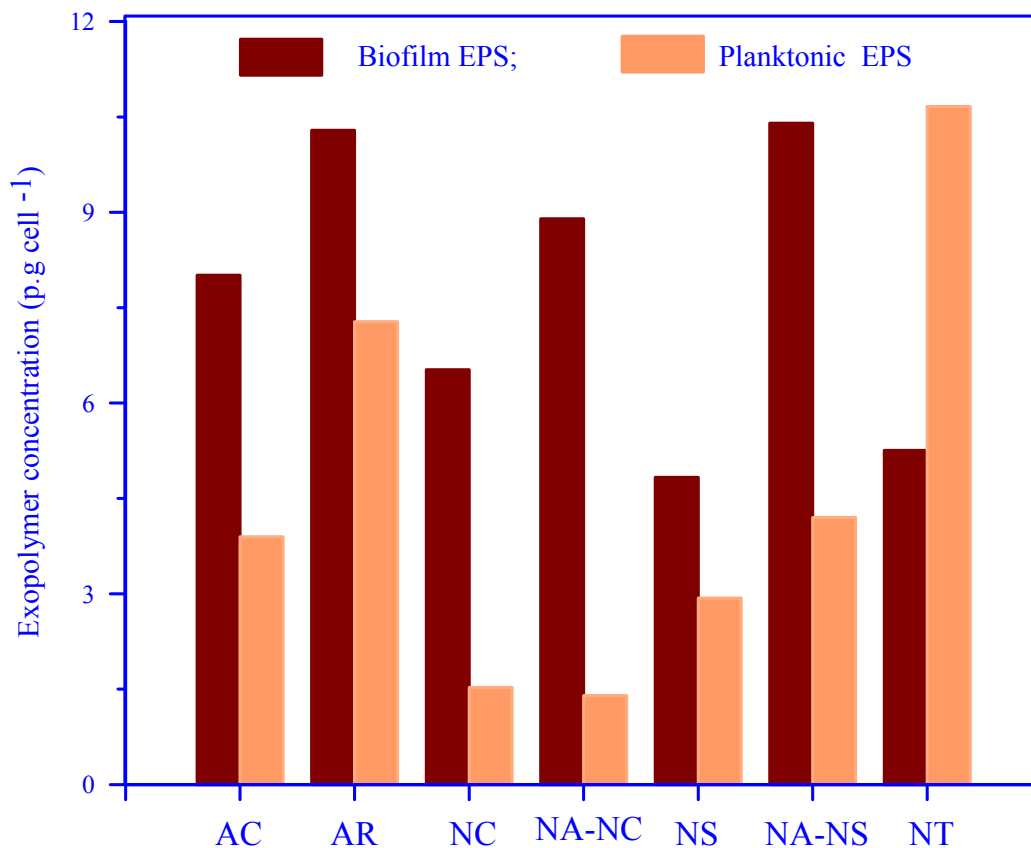


and homogeneity of variances before subjecting to statistical analysis. The influence of biofilm EPM, free EPM and diatom films of varying densities of axenic and non-axenic cultures on cyprid metamorphosis was evaluated by two-way analysis of variance (ANOVA) with equal replication (Sokal and Rohlf 1981).

### 5.3. RESULTS

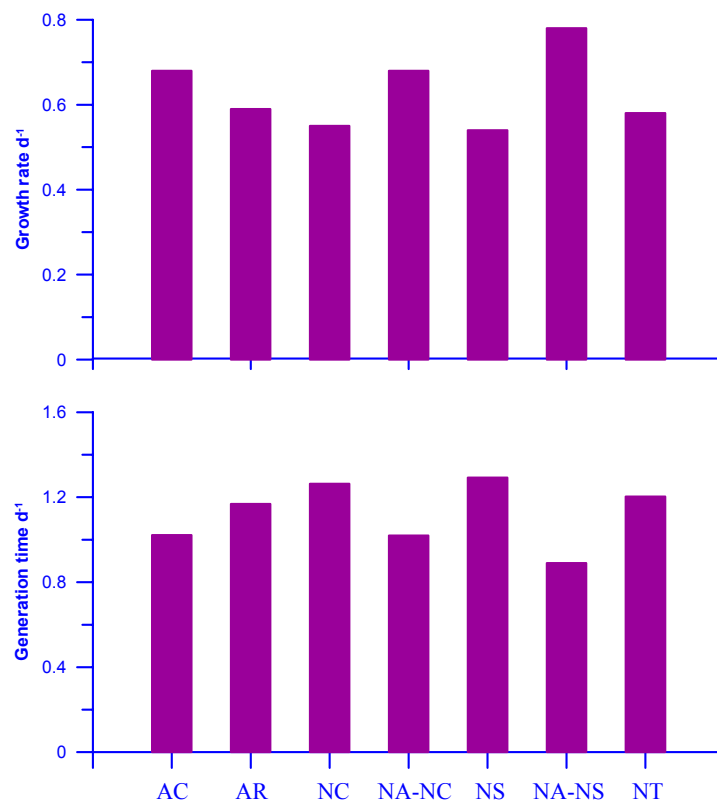
#### 5.3.1. Diatom growth and exopolymer production

Biofilm EPM was comparatively higher than free EPM, except for *N. transitans* var. *derasa* f. *delicatula* (Fig. 5.2). *Amphora* spp., which has faster growth rate and lesser generation time, produced higher biofilm exopolymers per cell compared to *Navicula* spp. (Figs. 5.2 and 5.3). Among the *Amphora* spp., *A. rostrata* produced higher EPM



**Fig. 5.2.** Exopolymer production by diatoms under axenic and non-axenic conditions. AC: *Amphora coffeaeformis*, AR: *A. rostrata*, NC: *Navicula crucicula*, ND: *N. transitans* var. *derasa* f. *delicatula*, NS: *N. subinflata*, NA-Nc: Non-axenic- *N. crucicula* and NA-NS: Non-axenic-*N. subinflata*.

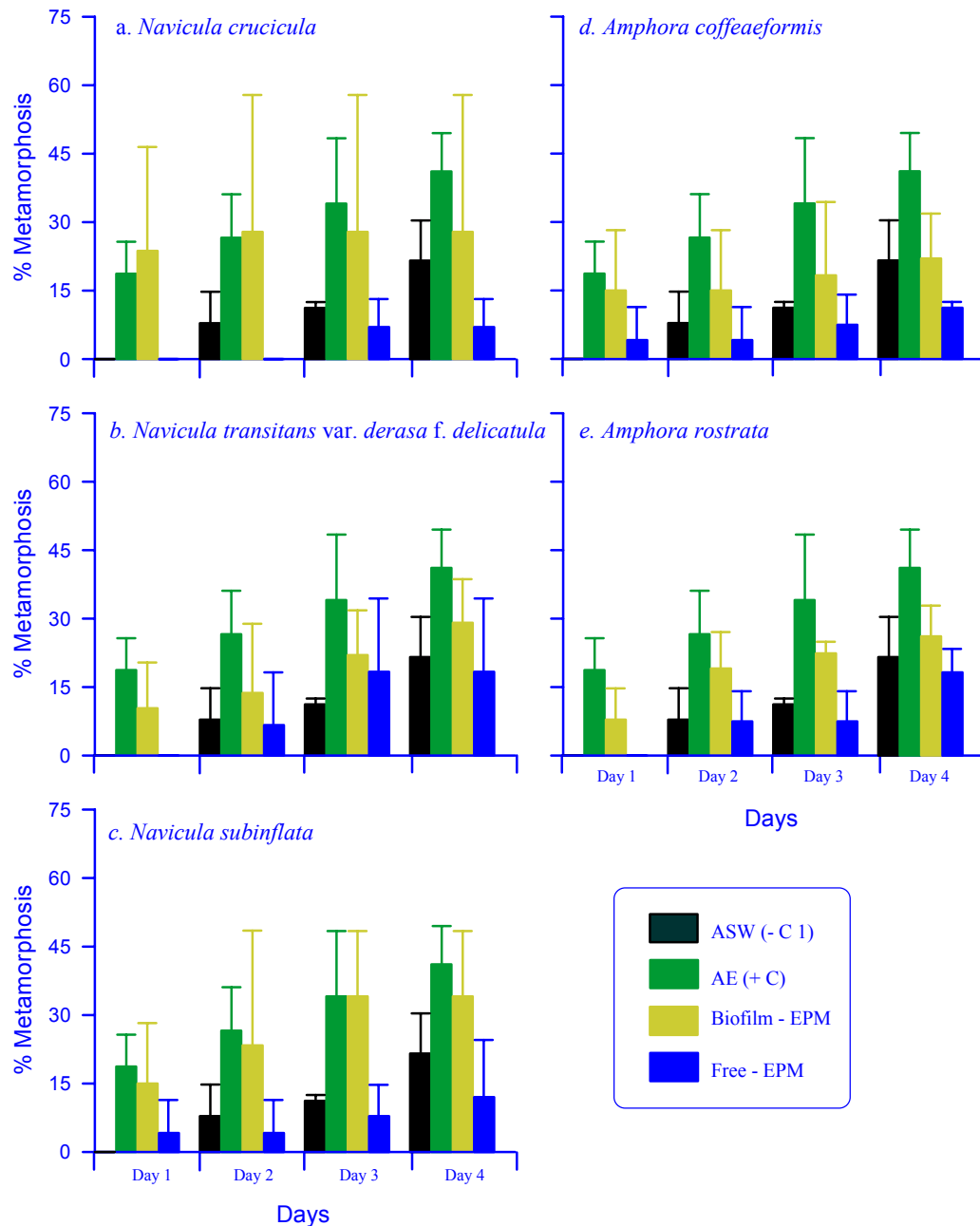
per cell followed by *A. coffeaeformis* (Fig. 5.2). In case of *Navicula* spp., the EPM production was in the following order; *N. crusicula* > *N. transitans* var. *derasa* f. *delicatula* > *N. subinflata* (Fig. 5.2). However, *N. transitans* var. *derasa* f. *delicatula* produced higher free EPM followed by *A. rostrata*, *A. coffeaeformis*, *N. subinflata* and *N. crusicula* (Fig. 5.2). In another set, it was found that the non-axenic culture of *N. subinflata* and *N. crusicula* produced higher biofilm EPM per cell than axenic cultures (Fig. 5.2). The results obtained from the growth rate also revealed that the growth rate of non-axenic diatom cultures was higher than axenic cultures (Fig. 5.3). This further indicates that the bacteria associated with the diatoms play an important role in the ecology of diatoms like growth rate and EPM production.



**Fig. 5.3.** Growth rate and generation time of diatoms grown under axenic and non-axenic conditions. AC: *Amphora coffeaeformis*, AR: *A. rostrata*, NC: *Navicula crusicula*, NT: *N. transitans* var. *derasa* f. *delicatula*, NS: *N. subinflata*, NA-NC: Non-axenic- *N. crusicula* and NA-NS: Non-axenic-*N. subinflata*.

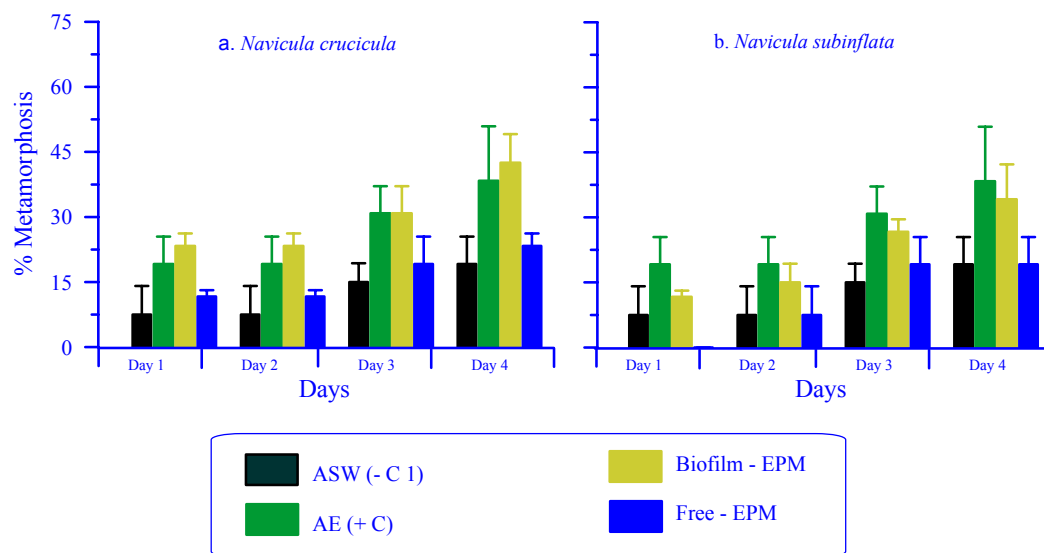
### 5.3.2. Effects of diatom exopolymers on cyprid metamorphosis

The results of the larval assays are shown in Fig. 5.4. The results obtained revealed that the diatom exopolymers did not promote larval settlement induction as compared to positive control (Fig. 5.4). Among the exopolymers, the maximum percentage of larval



**Fig. 5.4.** Influence of diatom exopolymers produced under axenic conditions on the larval metamorphosis of *B. amphitrite*. ASW (-C): Autoclaved sea water (- control) and AE(+C): Adult extract (+ control).

metamorphosis was shown for biofilm EPM than free EPM for all experimental days (Fig. 5.4). Two-way ANOVA showed significant variation between the exopolymers and controls (Table 5.1) which was in the following order; AE > biofilm-EPM > FSW (negative control) > Free EPM. Among the diatom genera, exopolymers of *Navicula* spp. showed higher rates of larval metamorphosis compared to *Amphora* spp. (Fig. 5.4). However, two-way ANOVA showed insignificant variations between the species (for both inter-generic or intra-generic) (Table 5.1). *Amphora* spp. yielded more exopolymer per cell but was less influential on larval metamorphosis than *Navicula* spp. and vice versa (Figs. 5.2 and 5.4). Among the non-axenic cultures, the biofilm EPM of *N. crusicula* showed higher metamorphosis compared to AE. However, the same was not the case with *N. subinflata* (Fig. 5.5). The results also revealed that EPM of non-axenic cultures of *N. crusicula* and *N. subinflata* promoted larval metamorphosis better than axenic cultures. However, two-way ANOVA showed insignificant variation between the axenic and non-axenic cultures but significant variation was observed between the assay conditions for all experimental days (Table 5.2).



**Fig. 5.5.** Influence of diatom exopolymers produced under non-axenic conditions on the larval metamorphosis of *B. amphitrite*. ASW (-C): Autoclaved sea water (- control) and AE(+C): Adult extract (+ control).

**Table 5.1.** Two-way ANOVA. Influence of exopolymers (biofilm/free) of axenic diatom species (AC, AR, NC, NT, NS) on the metamorphosis of *Balanus amphitrite* cyprids. AC-*A. coffeaeformis*, AR-*A. rostrata*, NC-*N. crusicula*, NT-*N. transitans* and NS-*N. subinflata*. (Note: \*\*\*\* -  $p \leq 0.001$ ; \*\*\* -  $p \leq 0.01$ ; \*\* -  $p \leq 0.025$ ; \* -  $p \leq 0.05$ ; ns – not significant).

Variations	df	Day 1			Day 2			Day 3			Day 4		
		SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>
A(Species)	4	47	11.7	0.49 <sup>ns</sup>	21	5.3	0.10 <sup>ns</sup>	50.0	12.5	0.23 <sup>ns</sup>	43	10.73	0.23 <sup>ns</sup>
B(Assay condition)	3	1294	431.4	18.16****	1640	546.8	10.17****	2170.6	723.5	13.20****	2225	741.57	15.61****
A*B (interaction)	12	132	11.0	0.46 <sup>ns</sup>	180	15.0	0.28 <sup>ns</sup>	219.1	18.3	0.33 <sup>ns</sup>	140	11.70	0.25 <sup>ns</sup>
within subgroup error	40	950	23.8		2150	53.8		2191.7	54.8	0.33	1900	47.51	
Total	59	2424			3992			4631.3			4308		

**Table 5.2.** Two-way ANOVA. Influence of exopolymers (biofilm and free) of axenic and non-axenic diatom species (NC and NS) on the metamorphosis of *Balanus amphitrite* cyprids. NC-*N. crusicula* and NS-*N. subinflata*. (Note: \*\*\*\* -  $p \leq 0.001$ ; \*\*\* -  $p \leq 0.01$ ; \*\* -  $p \leq 0.025$ ; \* -  $p \leq 0.05$ ; ns – not significant)

Variations	df	Day 1			Day 2			Day 3			Day 4		
		SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>
A(cultures)	3	94	31.3	1.39 <sup>ns</sup>	58	19.3	0.40 <sup>ns</sup>	27	8.9	0.20 <sup>ns</sup>	81	27.0	0.53 <sup>ns</sup>
B(Assay condition)	3	890	296.8	13.18****	1000	333.4	6.89****	1393	464.2	10.53****	1746	581.9	11.44****
A*B (interaction)	9	169	18.7	0.83 <sup>ns</sup>	218	24.2	0.5 <sup>ns</sup>	174	19.3	0.44 <sup>ns</sup>	198	22.1	0.43 <sup>ns</sup>
within subgroup error	32	721	22.5		1549	48		1411	44.1		1628	50.87	
Total	47	1874			2824.8			3004			3653		

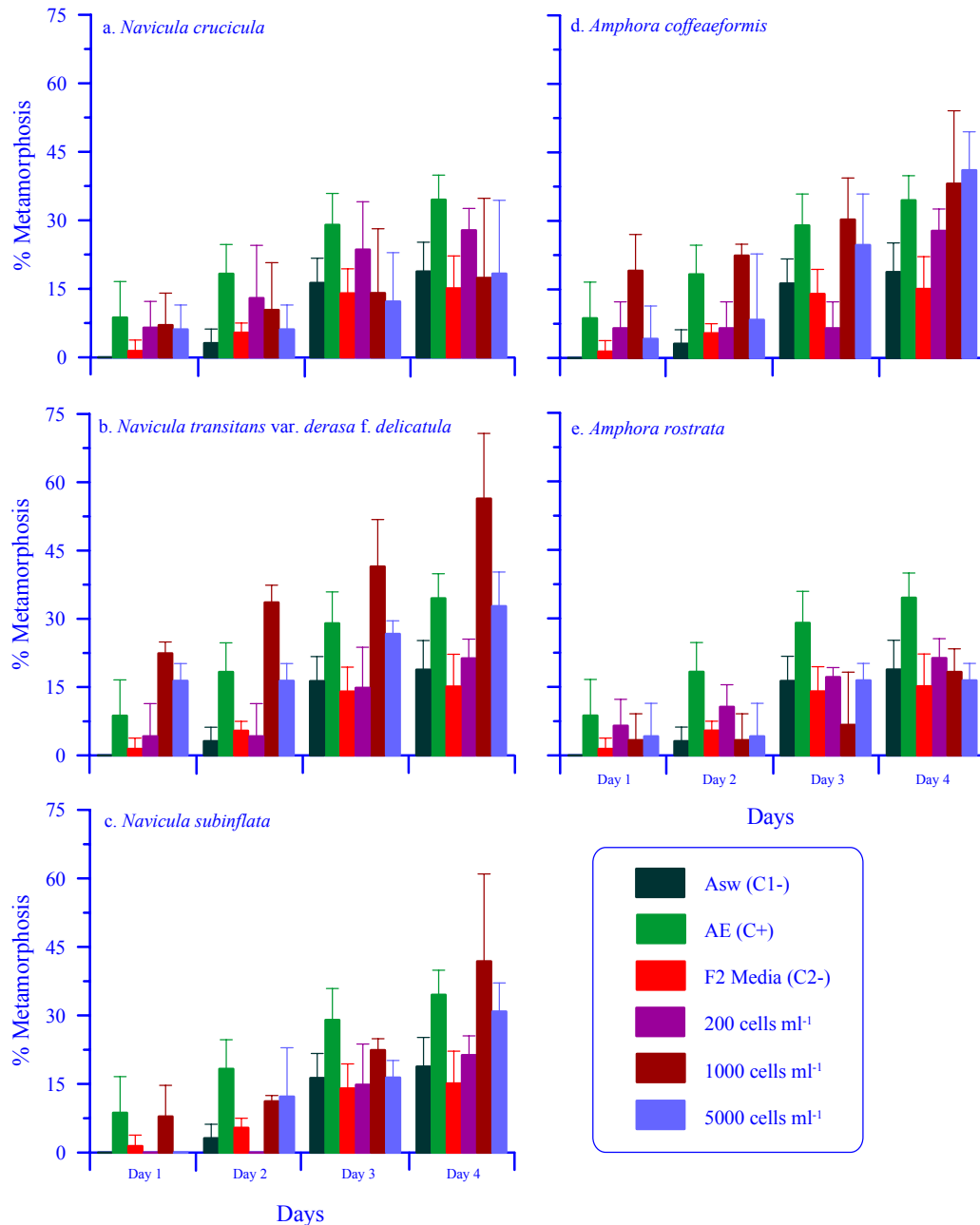
### 5.3.3. Effects of diatom films and its growth on cyprid metamorphosis

The results of larval assays are shown in Fig. 5.6. In the cases of *N. transitans* var. *derasa* f. *delicatula*, *N. subinflata* and *A. coffeaeformis*, the metamorphosis induction was better than the positive control. Such a promotion was not seen in the case of *N. crusicula* and *A. rostrata*. (Fig. 5.6). Two-way ANOVA showed significant variation between the assay conditions i.e. diatom films and controls (Table 5.3). The metamorphosis promotion by the diatom films was in the order of *N. transitans* var. *derasa* f. *delicatula* > *A. coffeaeformis* > *N. subinflata* (Fig. 5.6). Two-way ANOVA

**Table 5.3.** Two-way ANOVA. Influence of monospecific films of axenic diatom species (AC, AR, NC, NT, NS) at different cell densities (200, 1000, 5000) on the metamorphosis of *Balanus amphitrite* cyprids. AC-*A. coffeaeformis*, AR-*A. rostrata*, NC-*N. crusicula*, NT-*N. transitans* and NS-*N. subinflata*. (Note: \*\*\*\* -  $p \leq 0.001$ ; \*\*\* -  $p \leq 0.01$ ; \*\* -  $p \leq 0.025$ ; \* -  $p \leq 0.05$ ; ns – not significant)

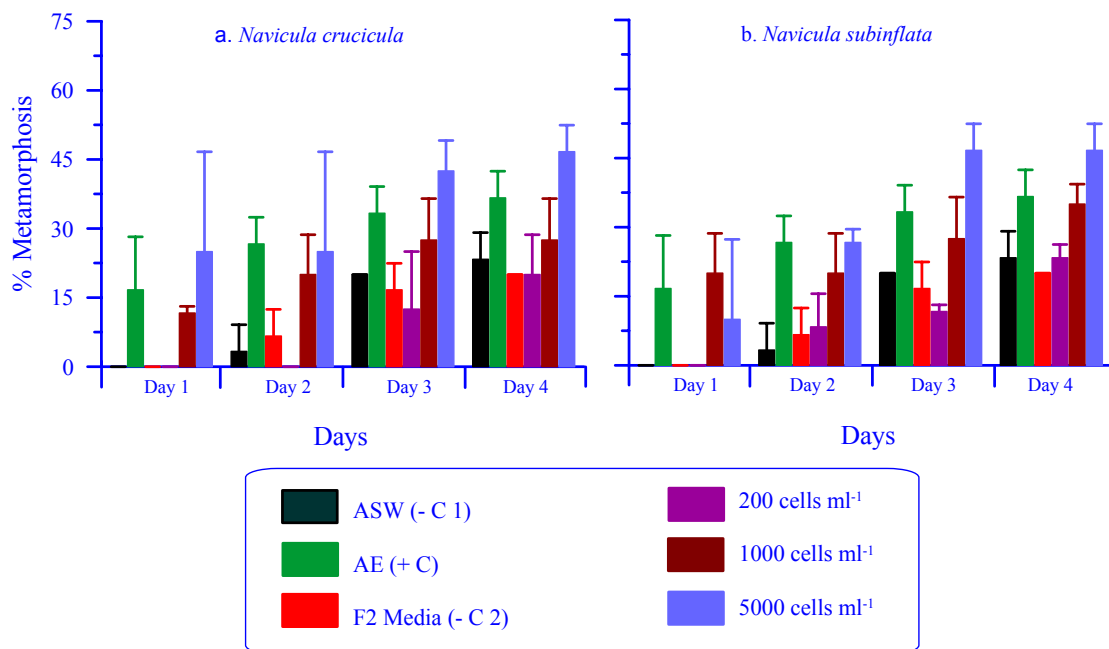
Variations	df	Day 1			Day 2			Day 3			Day 4		
		SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>	SS	MS	F <sub>s</sub>
A(Species)	4	127.6	31.9	2.87*	135.0	33.7	1.87 <sup>ns</sup>	184.4	46.1	1.57 <sup>ns</sup>	478.0	119.5	3.7***
B(Assay condition)	5	378.3	75.7	6.82****	587.1	117.4	6.50****	536.9	107.4	3.65****	1944.1	388.8	12.05****
A*B (interaction)	20	330.2	16.5	1.49 <sup>ns</sup>	648.6	32.4	1.8 <sup>ns</sup>	913.9	45.7	1.55 <sup>ns</sup>	1370.6	68.5	2.12**
within subgroup error	60	666.0	11.1		1083.1	18.1		1766.2	29.4		1936.6	32.3	
Total	89	1502.1			2453.8			3401.4			5729.3		

showed significant variation between the species on day one and four (Table 5.3). In case of *N. transitans* var. *derasa* f. *delicatula* and *N. subinflata*, higher metamorphosis was observed in the wells inoculated with 1000 cells ml<sup>-1</sup> than 200 and 5000 cells ml<sup>-1</sup> (Fig. 5.6). Whereas, in case of *A. coffeaeformis*, larval metamorphosis increased from



**Fig. 5.6.** Influence of axenic diatom films at three different densities on the larval metamorphosis of *B. amphitrite*. ASW (-C): Autoclaved sea water (- control), and AE(+C): Adult extract (+ control).

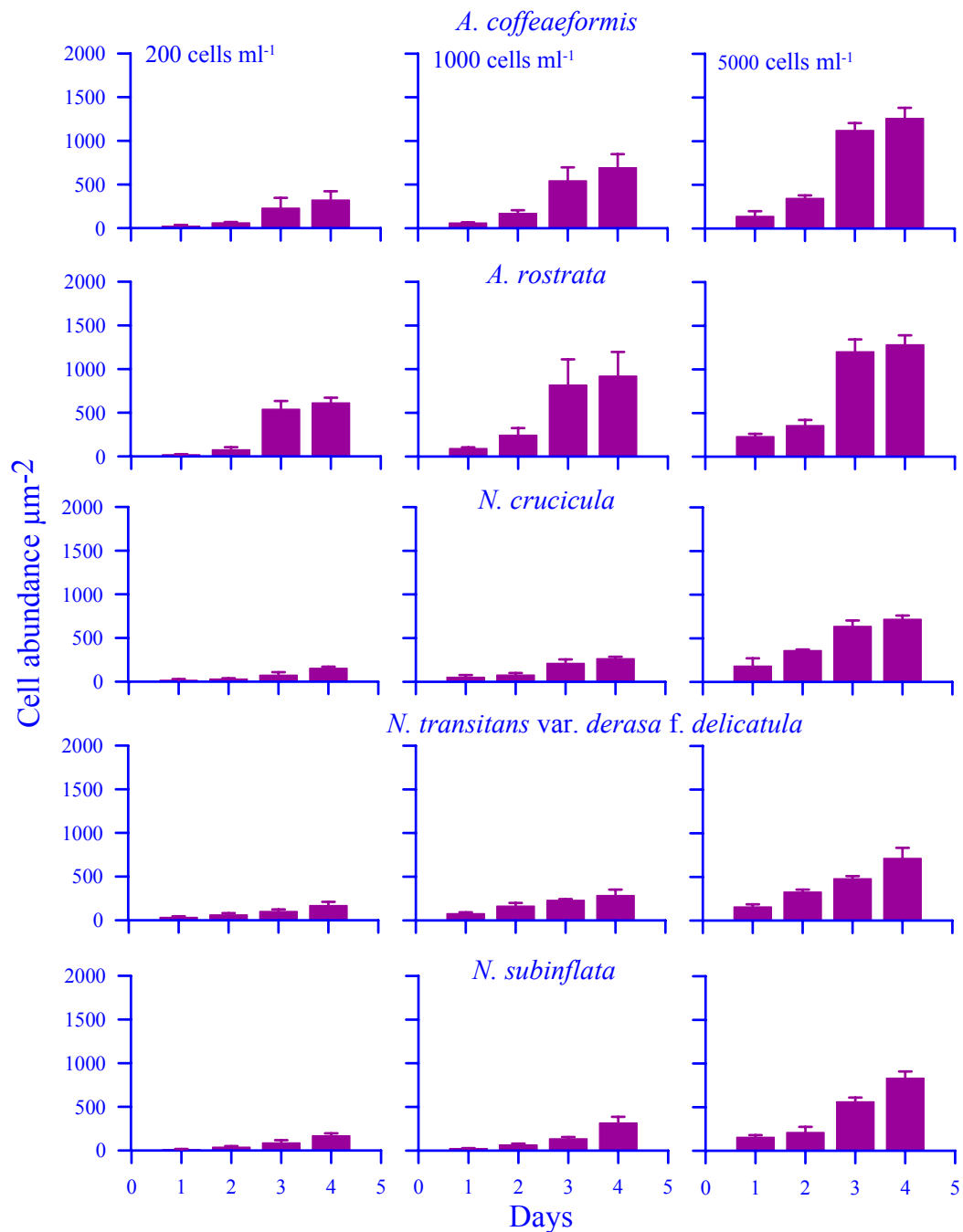
day 1 to day 4 and on day 4 the % metamorphosis observed fell in the following order 200>1000>5000 cells ml<sup>-1</sup> respectively. These results confirm that larval metamorphosis is species and density dependent. Assays with films of non-axenic diatoms revealed that the larval metamorphosis increased with cell density (Fig. 5.7). Compared with films of axenic diatoms, films of non-axenic diatoms promoted larval metamorphosis (Figs. 5.6 and 5.7). Two-way ANOVA showed significant variations between axenic and non-axenic diatoms as well as between the films and controls (Table 5.4). Growth of diatoms



**Table 5.4.** Two-way ANOVA. Influence of monospecific films of axenic and non-axenic diatom species (NC and NS) at different cell densities (200, 1000, 5000) on the metamorphosis of *Balanus amphitrite* cyprids. NC-*N. crucicula*, and NS-*N. subinflata*. (Note: \*\*\*\* -  $p \leq 0.001$ ; \*\*\* -  $p \leq 0.01$ ; \*\* -  $p \leq 0.025$ ; \* -  $p \leq 0.05$ ; ns – not significant).

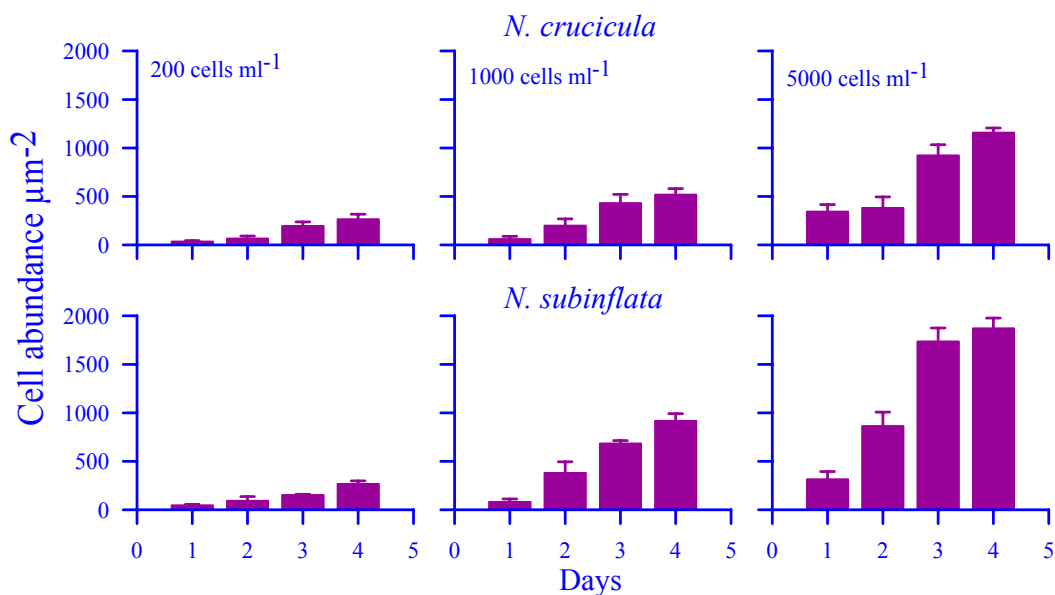
Variations	df	Day 1			Day 2			Day 3			Day 4		
		SS	MS	$F_S$	SS	MS	$F_S$	SS	MS	$F_S$	SS	MS	$F_S$
A(cultures)	3	148.9	49.6	2.39 <sup>ns</sup>	227	75.7	3.39 <sup>**</sup>	118	39.3	1.30 <sup>ns</sup>	394.4	131.5	5.07 <sup>***</sup>
B(Assay condition)	5	564	112.8	5.44 <sup>****</sup>	1031	206.2	9.23 <sup>****</sup>	658	131.6	4.36 <sup>***</sup>	1339.6	267.9	10.33 <sup>****</sup>
A*B (interaction)	15	516	34.4	1.66 <sup>ns</sup>	458	30.5	1.36 <sup>ns</sup>	621	41.4	1.37 <sup>ns</sup>	790.1	52.7	2.03 <sup>ns</sup>
within subgroup error	48	995	20.7		1072	22.3		1449	30.18		1244.7	25.9	
Total	71	2224			2788			2845			3768.7		

in the assay wells was also observed during the experiment with both axenic (Fig. 5.8) and non-axenic (Fig. 5.9) diatoms. Under axenic conditions the growth rate of *Amphora* spp. at all densities was higher as compared to that of *Navicula* spp. (Fig. 5.8). In case of axenic and non-axenic conditions the growth rate of *N. crucicula* and *N. subinflata* was higher in non-axenic conditions (Figs. 5.8 and 5.9).



**Fig. 5.8.** Growth of diatoms observed in the assay wells during the larval assays with the films of axenic diatom at three different densities.





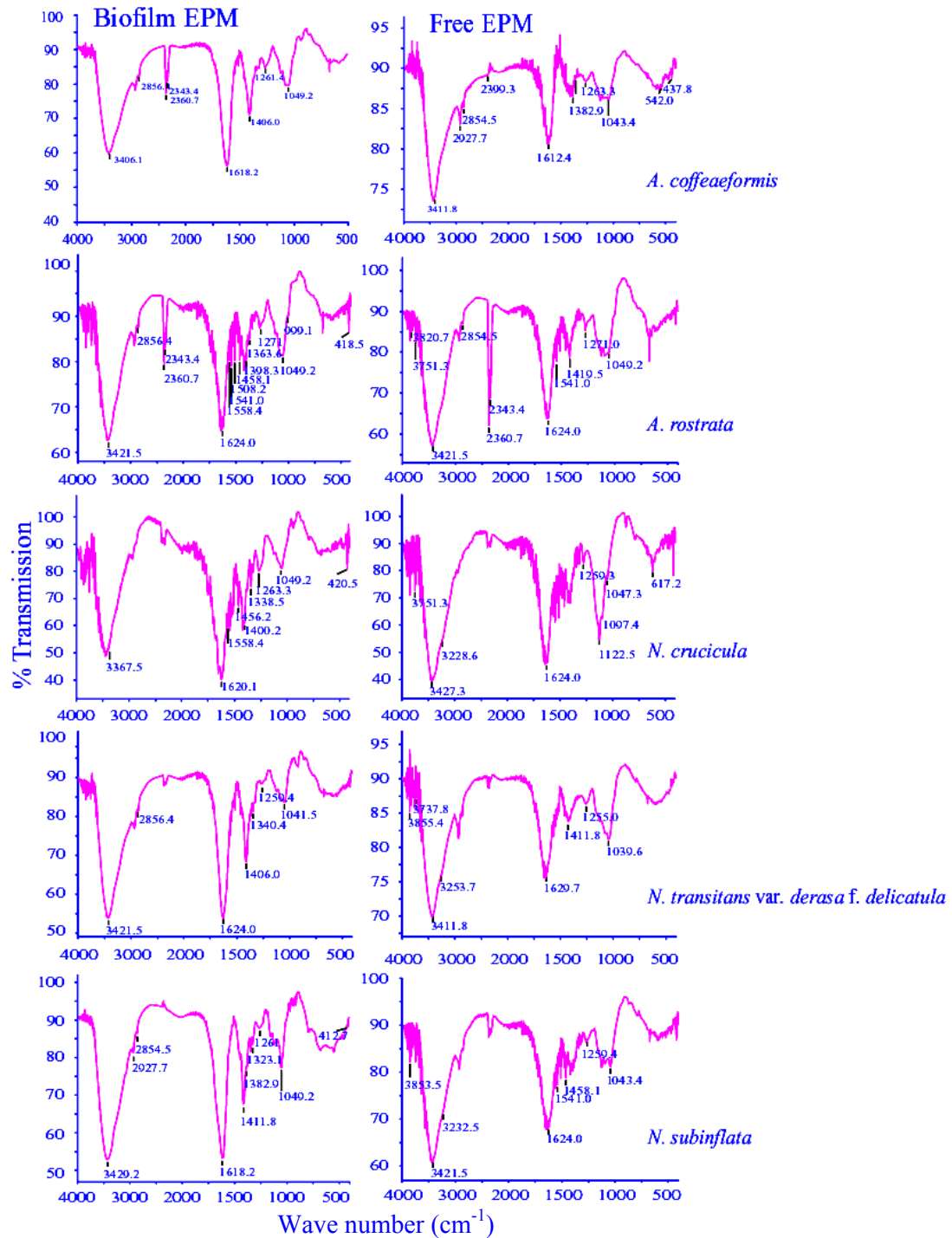
**Fig. 5.9.** Growth of diatoms observed in the assay wells during the larval assays with the films of non-axenic diatom at three different densities.

#### 5.3.4. FTIR analysis

The FTIR spectra of biofilm EPM and free EPM of the diatoms chosen for the study showed similar characteristic strong peaks around 3429 to 3367  $\text{cm}^{-1}$ , 1629 to 1612  $\text{cm}^{-1}$ , 1460 to 1406  $\text{cm}^{-1}$ , 1271 to 1259  $\text{cm}^{-1}$ , and 1122 to 1039  $\text{cm}^{-1}$  for O-H stretching, N-H bend, carboxylic and sulfate groups (Fig. 5.10) respectively. The structural identity of diatom EPM (both biofilm and free) from FTIR results was mainly of carbohydrates. However, the N-H bend indicates the presence of proteins.

## 5.4. DISCUSSION

Majority of diatoms that inhabit the submerged surfaces in illuminated environments are benthic, mostly pennate diatoms. Benthic diatoms are able to excrete copious amounts of extrapolymeric substances (EPS) that are primarily comprised of carbohydrates (Hoagland et al. 1993). These polymers play an important role in the ecology of intertidal mudflats and epibenthic communities. Benthic diatoms themselves probably benefit from the excretion of EPS in various ways, such as motility, attachment to surfaces, the ability to sequester toxic compounds, to capture nutrients and to protect



**Fig. 5.10.** Fourier transformed infrared spectroscopy (FTIR) spectra of the diatom exopolymers.

against desiccation (Decho 1990). For the ecosystem as a whole, excretion of EPS by benthic diatoms is important because it provides a food source for other organisms (van Duyl et al. 1999; Middelburg et al. 2000) and stabilizes the sediment surface (Paterson

1997). However, till date very little information is available on the cues offered by the biofilms dominated by benthic diatoms and its EPS for the settling invertebrate larvae. The present investigation was carried out to evaluate whether the films of monospecific benthic diatoms and its EPS generated a similar range of cues for the cyprid larvae of *Balanus amphitrite* as shown with bacteria and its components.

Altogether, 5 species of diatoms belonging to two genera viz. *Amphora* (*A. coffeaeformis* and *A. rostrata*) and *Navicula* (*N. crucicula*, *N. transitans* var. *derasa* f. *delicatula* and *N. subinflata*) dominant in the fouling community (Chapter 3) were chosen for the study. Amongst these, *N. transitans* var. *derasa* f. *delicatula* and *A. coffeaeformis* are also dominant in the microphytobenthic community and are the most important group of primary producers in the benthic ecosystem (Mitbavkar and Anil 2002). Part of the carbon that is fixed by benthic diatoms is excreted as extracellular polymeric substances, an important component of the diatom films (de Brouwer et al. 2000). However the amount of EPM production varies with the species in both, axenic and non-axenic cultures (Fig. 5.2). The diatoms chosen for the study produced maximum biofilm EPM compared to free EPM except for *N. transitans* var. *derasa* f. *delicatula* (Fig. 5.2). *Amphora* spp. (*A. rostrata* > *A. coffeaeformis*) yielded maximum biofilm EPM per cell than *Navicula* spp. (*N. crucicula* > *N. transitans* var. *derasa* f. *delicatula* > *N. subinflata*) (Fig. 5.2) indicating inter and intra-generic variations. Whereas, in case of free EPM production, *N. transitans* var. *derasa* f. *delicatula* yielded maximum, followed by *A. rostrata*, *A. coffeaeformis*, *N. subinflata* and *N. crucicula* (Fig. 5.2). Probably, *N. transitans* var. *derasa* f. *delicatula* and *A. coffeaeformis*, which are the most dominant forms in nature (Mitbavkar and Anil 2002) act as a source of dissolved carbon to the overlying waters. However, this aspect needs detailed investigation. In case of *N. crucicula* and *N. subinflata*, non-axenic cultures produced maximum biofilm EPM per

cell compared to axenic cultures (Fig. 5.2) indicating the contribution of bacteria in the production of EPM. Whereas, with respect to free EPM production, the rate was same with both, axenic and non-axenic cultures except for *N. subinflata* (Fig. 5.2). The amount of polymer extracted and composition again depends upon various factors like environmental conditions (Staats et al. 1999, 2000) and the method employed for the extraction of cellular EPM (Bhosle et al. 1995, 1996, Staats et al. 1999, Khandeparker and Bhosle 2001). A number of methods have been used for the extraction of EPM from cells in culture (Staats et al. 1999, Bhosle et al. 1995, Khandeparker and Bhosle 2001, De Brouwer et al. 2002). In the present study, isotonic buffer was used for the extraction of biofilm exopolymers as suggested by Beech et al. (2000). The buffer is used primarily to prevent cell rupture due to change in pH and also helps in extracting the capsular EPS with minimal cell damage.

In the present study, the results obtained from the larval assays in comparison to positive and negative controls, categorized the settlement responses into 3 distinct groups, i.e. 1. 'Inductive': percentage of metamorphosis is greater or equal to positive control, 2. 'Weakly inductive': percentage of metamorphosis is greater than negative control but less than positive control, and 3. 'No effect': percentage of metamorphosis is equal to or less than negative control. This classification is done in a similar fashion as described by Harder et al. (2002) for the larvae of fouling polychaete, *Hydroides elegans* Haswell (1883). The results revealed that the biofilm EPM produced by the diatoms represented category (2) whereas planktonic EPM represented category (3) with an inductive effect on larval settlement of *B. amphitrite* (Fig. 5.4). Two-way ANOVA showed significant variation between the controls and EPM (Table 5.1). This indicates that even though the diatoms produce enormous quantity of EPM they did not promote strongly the larval metamorphosis like in several bacterial strains. In the case of bacteria, the inductive

effect has been attributed to bacterial secondary metabolites and glycoproteinacious bacterial exopolymers (Unabia and Hadfield 1999). Similar conclusions were drawn with respect to the induction of larval settlement by natural biofilms in other invertebrate taxa (Zobell and Allen 1935, Kirchmann et al. 1982, Johnson et al. 1991, Szewzyk et al. 1991, Leitz and Wagner 1993, Maki 1999). This indicates that biofilm comprising much of diatom EPS may give weak positive cues for the incoming larvae. Among the diatoms, *Amphora* spp. yielded more of biofilm EPM per cell but was less influential on larval metamorphosis than *Navicula* spp. and vice versa. However, two-way ANOVA showed insignificant variation between the species (Table 5.1). In case of non-axenic diatoms, the biofilm and free EPM of *N. crucicula* represented category (1) and (2) respectively (Fig. 5.5), whereas in the case of *N. subinflata*, the biofilm and free EPM represented category (2) and (3) (Fig. 5.5) respectively. This indicates that the biofilm EPM produced by non-axenic diatoms facilitated larval metamorphosis from weak to strong whereas EPM of axenic diatoms exhibited from no effect to weak effect. Further suggesting that the diatoms in association with bacteria not only influenced the enhanced production of EPM but also mediated larval metamorphosis. Literature suggests that the percentage composition of the monosaccharides in the exopolymer produced by diatoms differed with species (Table 5.5). A summary of the monosaccharide composition of some diatoms selected in the study based on the available literature is shown in Table 5.5. The monosaccharide composition of the diatom EPM revealed that the glucose is most dominant in the EPM produced by *Navicula subinflata* whereas in case of *Amphora* spp., glucose, galactose, mannose and xylose are dominant (Table 5.5).

Neal and Yule (1996) considered that the structure of bacterial exopolymers is capable of either determining the effectiveness of the cyprids temporary adhesive or affecting the cyprid's willingness to detach (Yule and Walker 1984). This indicates that the

**Table 5.5.** Summary of the monosaccharide composition of the diatom EPM based on the literature. a: Wustman et. al. 1997, b: Bhosle et. al. 1996, c: Khandeparker & Bhosle 2001 and d: Bhosle et. al. 1995.

Organisms	Monosaccharides						
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
<i>Amphora coffeaeformis</i> <sup>a</sup>							
Biofilm EPS							
Mechanically isolated capsules	9.0	9.0	-	9.0	13.0	13.0	40.0
water soluble material	8.0	31.0	2.0	8.0	3.0	26.0	20.0
water insoluble material	13.0	32.0	-	13.0	5.0	32.0	5.0
<i>Amphora coffeaeformis</i> <sup>b</sup>							
Capsular EPS	0.5	-	-	-	11.9	4.8	81.8
<i>Amphora rostrata</i> <sup>c</sup>							
Biofilm EPS	7.4	36.7	8.7	2.1	8.7	27.6	8.7
Free EPS	7.6	41.0	4.3	2.3	9.1	32.0	3.3
<i>Navicula subinflata</i> <sup>d</sup>							
Biofilm EPS	0.5	0.4	0.2	0.8	1.7	2.2	94.0

difference in the chemical composition of the exopolymer produced by different species of benthic diatoms in the biofilm may influence the effectiveness of the cyprid metamorphosis. FTIR spectra of the diatom EPM appears similar for all the diatom species i.e. it did not reveal inter or intra-generic variation (Fig. 5.10). This indicates that EPM produced by diatoms are of similar nature i.e. carbohydrates and proteins. However, the percentage composition varies with the species (Table 5.5), which might have influenced the rate of metamorphosis among the diatom species. Earlier observations have hypothesized that sugars in solution adsorb electrostatically through –OH groups to polar groups associated with the cyprid larva temporary adhesive (CTA) (Yule and Walker 1987). *Lens culinaris* agglutinin (LCA) – binding sugar chains (i.e. D-glucose and D-mannose) of the adult extract (AE) have been suggested to be involved in the settlement of *B. amphitrite* (Matsumura et. al. 1998). Khandeparker et. al. (2002) reported that the D-mannose sugar treated cyprids triggered metamorphosis but the LCA-specific sugars, D-glucose and D-galactose showed reduced effect. Based on these earlier reports, it is possible to infer that the qualitative and quantitative variations in the exopolymers produced by the diatoms can yield different cues.

The experiments with diatom films at different densities revealed that the larval metamorphosis is species and density dependent (Figs. 5.6 and 5.7). The films of diatoms, *N. transitans* var. *derasa* f. *delicatula*, *N. subinflata* and *A. coffeaeformis*, inoculated with 1000 and 5000 cells ml<sup>-1</sup> in the assay wells represented category (1) whereas that of 200 cells ml<sup>-1</sup> represented category (2) with reference to inductive effect on larval settlement of *B. amphitrite* (Fig. 5.6). This indicates that these species, which are dominant in the natural biofilm (Chapter 3, Mitbavkar and Anil 2002), can play an important role in the population dynamics of *B. amphitrite* by influencing the induction of larval settlement. *A. rostrata* and *N. crucicula* at all densities represented category (2) with an inductive effect on larval settlement of *B. amphitrite* (Fig. 5.6). ANOVA showed significant variation between the controls and cell densities for all experimental days whereas between species, the significant variation was observed for experimental day one and four (Table 5.3). In case of non-axenic cultures of *N. crucicula* and *N. subinflata*, the wells inoculated with 5000 cells ml<sup>-1</sup> represented category (1) by day 4 whereas wells inoculated with 200 and 1000 cells ml<sup>-1</sup> represented category (2) (Fig. 5.7) respectively. This further indicates that the larval metamorphosis increased with increase in cell density (Fig. 5.7). However, in comparison with axenic films, non-axenic films promoted larval metamorphosis (Figs. 5.6 a, c and 8) indicating the influence of surface associated bacteria in combination with diatoms on the larval induction.

The difference in growth rate observed for different species under axenic and non-axenic conditions (Figs. 5.3, 5.8 and 5.9) might have resulted in micro-scale variations on the surface of the assay wells. The diatoms used in the study have varying cell size, shape and behaviour (aggregation and spreading) (Table 5.6). *N. transitans* var. *derasa* f. *delicatula*, *N. subinflata* and *A. coffeaeformis* initially formed the film by

**Table 5.6.** Size, shape and nature of the film observed during investigation for the diatom used in the study.

Species	Size ( $\mu$ )		Shape	Film type
	L	B		
<i>Amphora coffeaeformis</i>	23	9	Cymbelloid	Aggregate
<i>Amphora rostrata</i>	57	17	Cymbelloid	Uniform
<i>Navicula crucicula</i>	10	3	Prism on elliptical base	Uniform
<i>Navicula transitans</i> var. <i>derasa</i> f. <i>delicatula</i>	40	11	Prism on elliptical base	Aggregate
<i>Navicula subinflata</i>	17	3	Prism on elliptical base	Aggregate

spreading uniformly and later on the cells aggregated into small colonies whereas *A. rostrata* (largest in size) and *N. crucicula* (smallest in size) developed an uniform film. The induction of larval metamorphosis was higher for former than latter (Fig. 5.6). Hence the observed micro-scale variations in the growth pattern exhibited by an individual species population might play an important role in the population dynamics of *B. amphitrite*.

In conclusion, larval assays revealed that the benthic diatom films and their components induced the metamorphosis in the following order: diatom films > biofilm EPS > free EPS for axenic diatoms suggesting that the cues offered by benthic diatoms can also mediate invertebrate larval metamorphosis. In the case of non-axenic diatoms, films and its biofilm components also promoted larval metamorphosis. However, the cues varied from weak to strong depending upon the species and their density in the films as well as the composition of extracellular polymeric substances. This investigation indicates that the bacteria associated with diatoms play an important role in providing cues to invertebrate larvae.