

Chapter 4

Modulations in the periphytic diatom diversity

4.1 Introduction

Periphytic diatoms in the littoral zone originate from a number of sources, including the water column, adjacent surfaces and benthos. Once dislodged, any substratum in the pelagic zone serves as an "island" for immigration to these tychoplanktonic cells. Ecologically, the pennate diatoms are important from two perspectives: first and foremost, they are important components of the microphytobenthic community, in both sandy and muddy intertidal flats (Round and Palmer 1966; Round 1979a, b; Round 1981). In addition to being a source of energy and nutrients to the benthic food web, they supply organic carbon to the plankton system during resuspension by tidal currents and wind-induced turbulence (de Jonge 1980; de Jonge 1985; de Jonge and Van Beusekom 1992; de Jonge and Colijn 1994; de Jonge and Van Beusekom 1995; de Jonge 1995). Secondly, when displaced from their original habitat, into the water column, these forms on encountering any kind of substratum tend to settle and develop a community of their own and are responsible for a major input of energy in the form of reduced carbon to the surface (Cooksey et al. 1984). The attached diatom community also constitutes a system rich in information for environmental monitoring, which can be exploited through analysis of communities' structural characteristics (Gold et al. 2002). Periphytic diatom communities have been used as biological indicators of river acidification (Ter Braak and Van Dam 1989), organic pollution (Desey and Coste 1991) and eutrophication (Kelly and Whitton 1995).

Space is a resource of utmost importance for the settlement and growth of the marine periphytic diatom community. However, limited availability of space as compared to the vast diversity of species, leads to intense competition for this resource. Thus, diversity is controlled by the competitive strategies employed by each member of the community, whether a pioneer or a late arrival. In this struggle for existence, the

fittest species can carve a better niche for itself, by exhibiting competitive traits. Valiela (1984) stated that competition occurs when two species require a resource that is in short supply, so that availability of that resource to one species is negatively influenced by the presence of other species, wherein the more competitive species

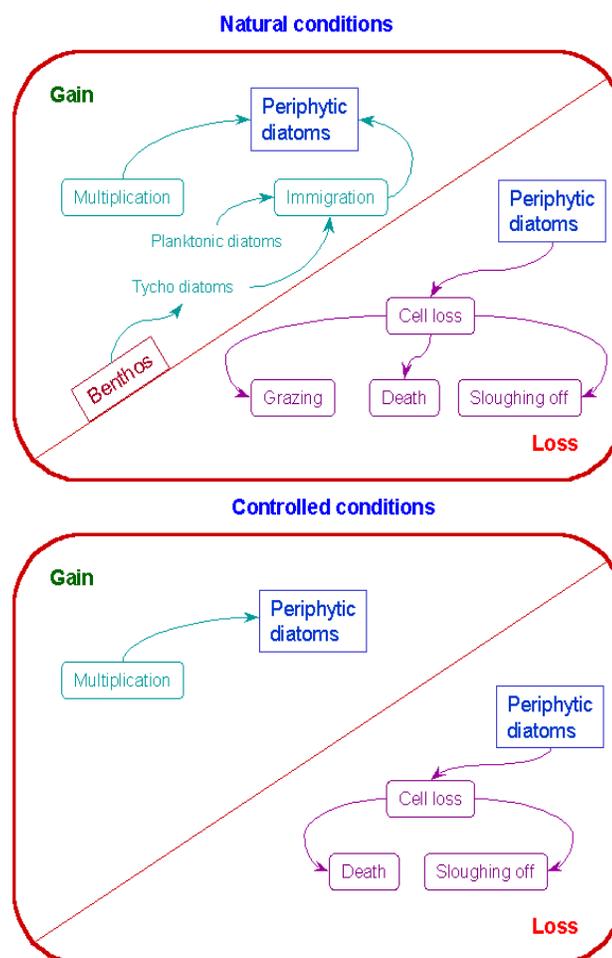


Fig. 4.1. Schematic representation of the processes governing the periphytic diatom community

proliferates. Ecosystem processes are governed by the biodiversity within its community and the gain or loss factors as illustrated in Fig. 4.1. Immigration and multiplication are the gain factors whereas grazing, sloughing off or death, are the loss factors influencing the periphytic diatom community in nature. Incubation of the

population from nature in the laboratory minimizes the gain factors only to multiplication and eliminates the grazing component from the loss factors.

In a homogenous habitat, species differ in their competitive abilities and are limited by and compete for the same single resource. According to Tilman (1982, 1999), in such a habitat at equilibrium, the best competitor, among the species present would win. In this effort, we subjected the diatom films developed on glass surface in the marine environment to incubation under controlled conditions. Through this, we illustrate the roles of seeding, nutrients and competitive strategies in the variations of marine periphytic diatom diversity.

4.2 Materials and methods

4.2.1 Experimental design

Glass slides (measuring 7.6 x 2.2 cm) were fixed on to a wooden frame, which was suspended at the sub-surface level (~ 1m below the low tide level so as to ensure continuous immersion in seawater) at the Dona Paula Bay (15⁰ 27' N, 73⁰ 48' E), Goa, west coast of India. A total of four glass slides were retrieved every consecutive day, for a period of four days in order to obtain 1, 2, 3 and 4 days old diatom community. Earlier reports (Smita et al. 1997; Smita and Anil 2000) and personal observations of the present study area have revealed the dominance of microorganisms on substrates for one to four days, followed by the arrival of macro organisms. As our aim was to understand the progression of a periphytic diatom community in the very early stages in an undisturbed condition, this study restricted the field exposures to only four days. After recovering from field, the slides were subjected to quantification and characterization of the diatom community.

4.2.2 Laboratory incubation

Out of the four slides, two were incubated in autoclaved nutrient enriched seawater (ES) (f/2, Guillard and Ryther 1962) whereas the other two were incubated in autoclaved seawater (AS) separately. The salinity of seawater ranged between 32 to 35 psu. The molar ratios of N:P:Si (μM) in ES were 882:42:123 and in AS were 1.3:1.8:25, 1.5:3.2:37 and 0.45:1.2:50 for post-monsoon, pre-monsoon and monsoon respectively. The slides were later used for direct diatom counts every alternate day

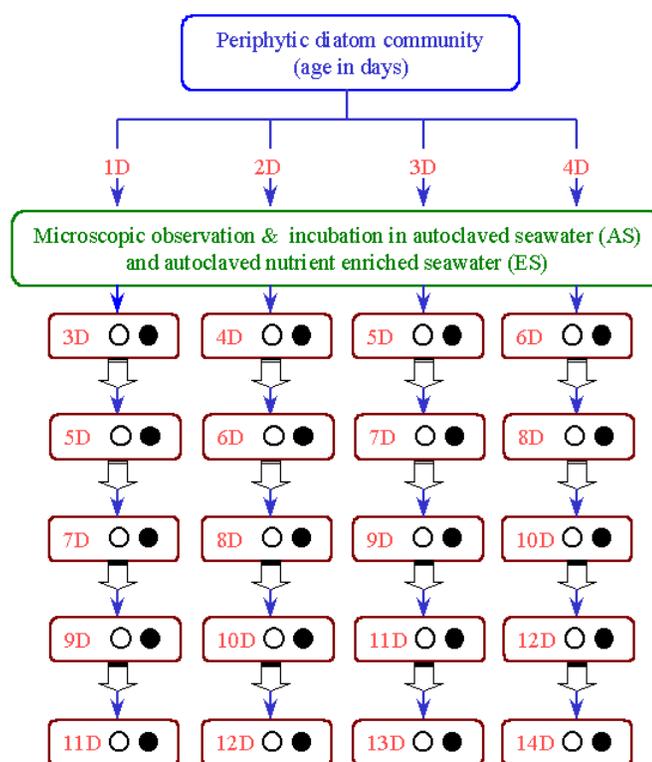


Fig. 4.2. Schematic representation of the experimental protocol (D = Days; O = Observation of glass slide; ● = Observation of sloughed off community; ⇄ = Transfer of glass slide to fresh medium)

and transferred into fresh ES and AS respectively. Counts were also taken of the cells lost from the slides into the media (Fig. 4.2). Incubation was carried out at 27°C , which was close to ambient temperatures. Slides were illuminated at an incident irradiance of $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, produced by fluorescent tubes over a 12 h light: 12 h dark photoperiod.

Earlier observations have shown a considerable variation in the diatom species diversity during the three seasons i.e., post-monsoon (January '99), pre-monsoon (May '99) and monsoon (September '99) experienced by this locality (Smita and Anil 2000). Hence this experiment was carried out thrice, once in each season.

4.2.3 Enumeration of diatoms

Diatoms were identified based on the keys provided by Heurck (1896), Subrahmanyam (1946), Desikachary (1977,1987) and Tomas (1997). The diatom flora on either surface of the slide was analyzed and is expressed in terms of their variation per dm². For the purpose of linear regression analysis, specific growth rates (K) of the dominant periphytic diatom species were calculated according to Carreto and Catoggio (1976).

$$K = (\log_{10} N - \log_{10} N_0) * 2.3 / t$$

Where N = Cell concentration for every second day

$$N_0 = \text{Cell concentration at } t = 0$$

$$t = \text{day}$$

Area of the periphytic diatom species encountered on glass slides was calculated in terms of cm² (Hillebrand et al. 1999; Table 4.1).

4.2.4 Competition between *N. delicatula* and *A. coffeaeformis* in culture

Isolation and culture of benthic diatoms: Glass slides were suspended for 5 days at the sub-surface level (approx. 1m below low tide level) at Dona Paula Bay. After retrieval, the slides were rinsed with filtered seawater and the biofilm was brushed off with sterile paintbrushes into 50 ml CORNING tubes containing filtered seawater. A dilution series of the biofilm suspension was inoculated onto 1% agar prepared with f/2 nutrient medium. Agar plates were incubated at 27 ± 1 °C under a 12 h light: 12 h dark photcycle. Distinguishable diatom colonies of *A. coffeaeformis* and *N.*

delicatula formed within 1 week of inoculation which were isolated and successively sub-cultured until monospecific diatoms were obtained. Pure cultures were then maintained in f/2 nutrient medium enriched seawater.

Experimental protocol: Pure diatom suspensions of each of the two species were prepared by brushing the culture flasks with sterile paintbrushes. Subsequently, aliquots of the two diatom suspensions were transferred into polystyrene multiwells (Corning-430343; 3 replicates) containing sterile f/2 medium. The initial inoculum density ratios of *N. delicatula* and *A. coffeaeformis* were (a) 99:1 (b) 95:5 (c) 85:15 (d) 75:25 (e) 50:50 (f) 25:75 (g) 15:85 (h) 5:95 and (i) 1:99. The final concentration was 50,000 cells ml⁻¹. These multiwells were incubated at 27 ± 1 °C under 12h light: 12h dark condition and were observed microscopically at regular intervals for quantification. Results are presented in terms of cells mm⁻². Specific growth rates of the two diatom species were calculated according to Carreto and Catoggio (1976).

4.2.5 Data analysis

Univariate analysis: Univariate techniques included the calculation of Shannon-Wiener diversity index (H') (Pielou 1969).

Multivariate analysis: A similarity matrix for the square root transformed diatom abundance data was constructed using the Bray-Curtis measure (Bray and Curtis 1957), which was then subjected to ordination analysis. Ordination was by non-metric multidimensional scaling (NMDS) (Kruskal and Wish 1978) using bubble plots. Univariate and multivariate analysis were made using PRIMER software version 5.

Linear regression analysis was performed on the specific growth rates of the dominant periphytic diatom species. Polynomial regression analysis was performed on the species diversity and percent coverage by the periphytic diatoms.

4.3 RESULTS

4.3.1 Water column diatom

Diatom composition during the three occasions is shown in Table 4.1 and figure 4.3. *Navicula delicatula* was the most abundant diatom in post-monsoon and pre-monsoon. In monsoon, the major forms were *Skeletonema costatum* (62%) and *Thalassionema frauenfeldii* (27%). Comparably high numbers of *Coscinodiscus marginatus* were present in post-monsoon, but this species decreased in abundance through pre-monsoon and monsoon. During post-monsoon and pre-monsoon, abundance of *Helicotheca tamesis* (8-13%) and *Pleurosigma angulatum* (5-17%) was greater than that in monsoon.

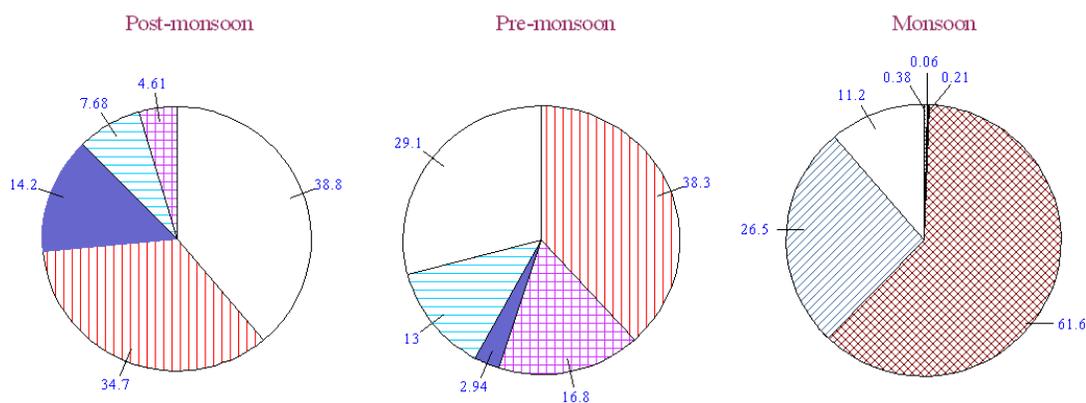


Fig. 4.3. Diatoms encountered in the ambient waters during post-monsoon, pre-monsoon and monsoon

- *Navicula delicatula*,
 - *Coscinodiscus marginatus*,
 - *Pleurosigma angulatum*,
 - *Skeletonema costatum*,
 - *Thalassionema frauenfeldii*,
 - *Helicotheca tamesis*,
 - Minors

Table 4.1 Diatoms encountered in case I, case II and case III and the area of periphytic diatoms

Pennate diatoms	Case I		Case II		Case III		Area of periphytic diatoms (x 10 ³ μm ²)
	WC	GL	WC	GL	WC	GL	
<i>Achnanthes longipes</i> Agardh	+			+		+	2.6
<i>Achnanthes subsessilis</i> Kutzing		+	+	+	+	+	0.8
<i>Amphora coffeaeformis</i> (Agardh) Kutzing	+	+		+		+	0.06
<i>Amphora hyalina</i> Kutzing		+	+	+		+	0.3
<i>Amphora rostrata</i> Wm. Smith		+				+	0.8
<i>Amphora turgida</i> Gregory		+	+	+		+	0.09
<i>Cocconeis scutellum</i> Ehrenberg	+	+	+	+		+	1.2
<i>Diploneis smithii</i> (de Brebisson) Wm. Smith					+	+	0.9
<i>Fragilariopsis oceanica</i> (Cleve) Hasle					+	+	0.1
<i>Grammatophora marina</i> Kutzing	+		+	+			1.4
<i>Grammatophora serpentina</i> Ehrenberg						+	1.9
<i>Licmophora flabellata</i> (Greville) Agardh						+	2.2
<i>Licmophora juergensii</i> Agardh	+	+	+			+	0.2
<i>Licmophora lingbyei</i> (Kutzing) Grunow		+					0.2
<i>Licmophora paradoxa</i> (Lyngbye) Agardh				+		+	0.26
<i>Meuniera membranacea</i> (Cleve) P. C. Silva comb. nov.						+	3.9
<i>Navicula transitrans</i> var. <i>derasa</i> f. <i>delicatula</i> Heimdal	+	+	+	+	+	+	0.7
<i>Navicula septentrionalis</i> (Grunow) Gran				+		+	0.16
<i>Navicula subinflata</i> Grunow				+	+	+	0.076
<i>Nitzschia angularis</i> Wm. Smith		+				+	3.6
<i>Nitzschia closterium</i> (Ehrenberg) W. Smith	+	+	+	+	+	+	0.24
<i>Nitzschia longissima</i> (Brebisson) Grunow		+			+	+	8.4
<i>Nitzschia sigma</i> (Kutzing) W. Smith						+	2.2
<i>Pleurosigma angulatum</i> Sensu Wm. Smith emend. Sterrunburg	+	+	+	+	+	+	2.1
<i>Pleurosigma elongatum</i> Wm. Smith	+		+		+		
<i>Pseudo-nitzschia seriata</i> (Cleve) H. Peragallo				+		+	0.69
<i>Surirella</i> sp.			+				
<i>Synedropsis gaillonii</i> Grunow					+	+	4.4
<i>Thalassionema frauenfeldii</i> (Grunow) Hallegraeff		+			+		0.76
<i>Thalassionema nitzschioides</i> (Grunow) Mereschkowsky		+				+	0.58
Centric diatoms							
<i>Biddulphia mobiliensis</i> (Bailey) Grunow	+				+		
<i>Biddulphia obtusa</i> (Kutzing) Ralfs in Pritchard		+		+			3.8
<i>Biddulphia regia</i> (M. Schultze) Ostenfeld						+	
<i>Biddulphia rhombus</i> (Ehrenberg) W. Smith	+		+	+		+	14.9
<i>Cerataulina</i> sp.	+						
<i>Chaetoceros</i> sp1	+					+	
<i>Chaetoceros</i> sp2	+					+	
<i>Coscinodiscus marginatus</i> Ehrenberg	+	+	+	+	+	+	4.3
<i>Coscinodiscus concinnus</i> Wm. Smith	+		+		+		
<i>Eucampia zodiacus</i> Ehrenberg	+						
<i>Guinardia striata</i> (Stolterfoth) Hasle com. nov.						+	
<i>Helicotheca tamesis</i> (Shrubsole) Ricard	+		+				
<i>Hyalodiscus stelliger</i> (Bailey) Mann				+			11.3
<i>Leptocylindrus danicus</i> Cleve						+	
<i>Melosira nummuloides</i> C. A. Agardh			+	+		+	0.47
<i>Skeletonema costatum</i> (Greville) Cleve						+	
<i>Thalassiosira eccentrica</i> (Ehrenberg) Cleve						+	6.1

.(WC: Water column, GL: Glass slide)

4.3.2 Field incubated slides prior to laboratory incubation

A progressive increase in periphytic diatom abundance on the field incubated slides from one to four days, was a common feature observed during the three occasions. The diatom cell abundance was highest in post-monsoon followed by monsoon and pre-monsoon (Appendix 4.1a-d to 4.6a-d). Periphytic diatoms encountered on the three occasions are presented in Table 4.1. Pennate genera were dominant throughout the study period. Amongst these, *N. delicatula* was the most dominant diatom.

4.3.3 Laboratory incubation of one to four days old periphytic diatom community after retrieval from field

Subsequent to laboratory incubation, the periphytic diatom abundance increased with time, the increase being higher in ES than AS. This trend was observed on all three occasions, except for the third and fourth day incubation in post-monsoon. A change in the total diatom cell abundance gradient from post-monsoon > monsoon > pre-monsoon (Appendix 4.1a-d to 4.6a-d) to monsoon > pre-monsoon > post-monsoon was observed subsequent to laboratory incubation (Appendix 4.1e-h to 4.6e-h).

The observations from post-monsoon, pre-monsoon and monsoon yielded three scenarios and these are presented as Case I (Intergeneric competition), Case II (Inter and Intrageneric competition) and Case III (Competitive exclusion or co-existence) respectively.

Case I (Intergeneric competition)

N. delicatula, *Amphora coffeaeformis*, *T. frauenfeldii*, *P. angulatum* and *Nitzschia closterium* were the dominant species of the one to four days old field periphytic diatom community (Appendix 4.1a-d, 4.2a-d, 4.7-10). *N. delicatula*, which was the most dominant diatom, continued to be so subsequent to laboratory incubation in ES even after a period of 11 days, in case of the one day old community (Fig. 4.5d, Appendix 4.2e, 4.7e-h). *A. coffeaeformis* and *N. delicatula* exhibited similar growth

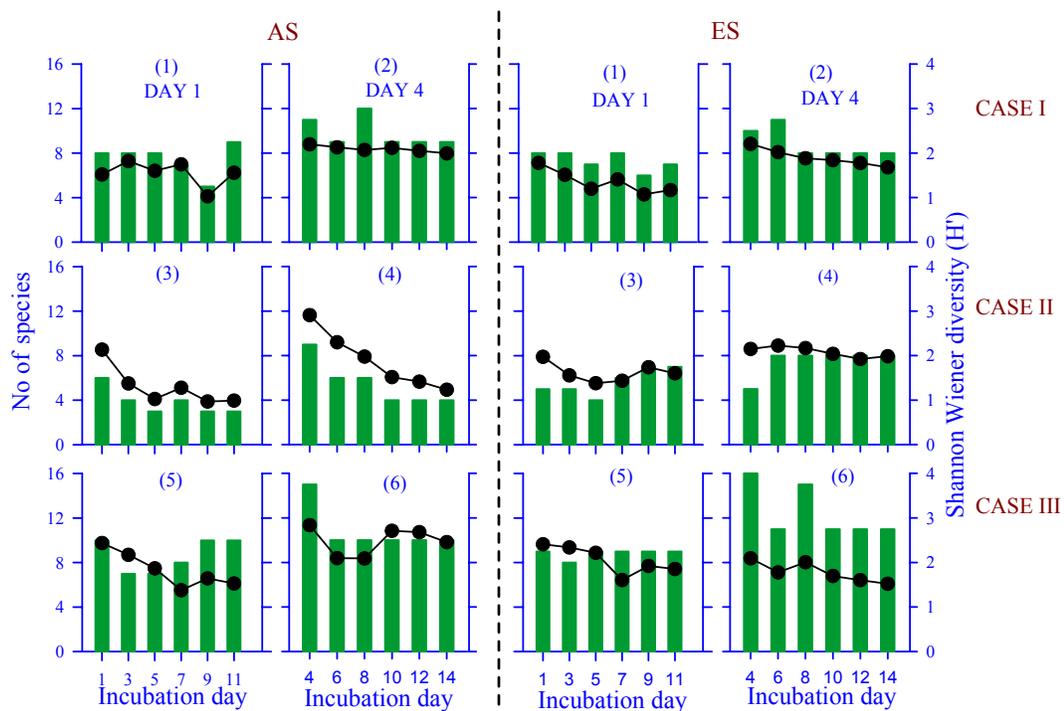


Fig. 4.4 Periphytic diatom species diversity in case I, case II and case III subsequent to laboratory incubation of one and four day old periphytic diatom community in AS and ES.

(Bars indicate number of species and • indicate species diversity)

rates in case of the incubation of two to three days old field communities in ES (Appendix 4.2f-g, 4.8g-l and 4.9g-l). In case of the four days old field community incubation in ES, the abundance of *A. coffeaeformis* increased at a faster rate, eventually surpassing *N. delicatula* with longer incubation period (Appendix 4.2h). This is reflected in the NMDS bubble plots (Fig. 4.5h, Appendix 4.10e-h) and resulted in a decreasing diversity profile (Fig. 4.4). Comparatively, in AS, the two major competitors were growing at almost the same pace (Fig. 4.5f and Appendix 4.1h, 4.7a-d to 4.10a-d). The community that had dislodged from the slide, in ES, had a meager population of *A. coffeaeformis* as compared to *N. delicatula* (Appendix 4.2i-l). Rest of the periphytic community which could not be in pace with the major competitors were also found to contribute to this sloughed off community. In AS, the two competitors, *N. delicatula* and *A. coffeaeformis* did not contribute much to this community while the other minor forms did (Appendix 4.1i-l). The *A. coffeaeformis* to *N. delicatula* abundance ratio in the initial field inoculum of the ES incubated four

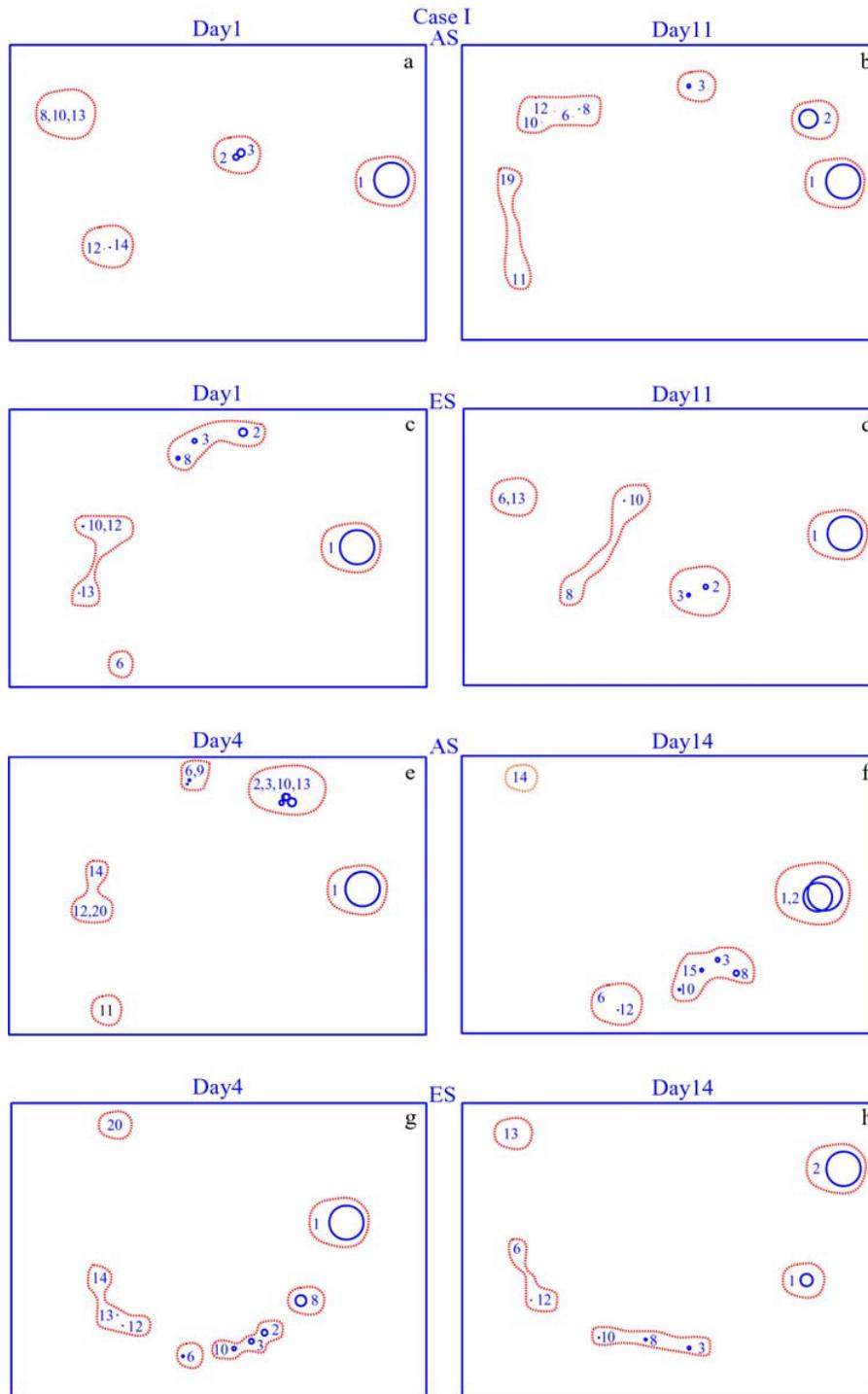


Fig. 4.5. Multidimensional scaling (MDS) ordinations for the case I periphytic diatom community incubated in AS and ES based on Bray-Curtis similarities. In the bubble plot, increasing size of circles indicate increasing abundance of a species (Stress = 0). Hatched lines indicate groups of species. 4.5a, c, d, e and g. *N. delicatula* (1) is the dominant diatom. 4.5b. *N. delicatula* (1) and *A. coffeaeformis* (2) are the dominant diatoms. 4.5f. *N. delicatula* (1) and *A. coffeaeformis* (2) exhibiting similar distribution. 4.5h. *A. coffeaeformis* (2) is the dominant diatom. 1. *N. delicatula*; 2. *A. coffeaeformis*; 3. *T. frauenfeldii*; 6. *N. longissima*; 8. *P. angulatum*; 9. *N. subinflata*; 10. *N. closterium*; 11. *C. marginatus*; 12. *C. scutellum*; 13. *N. angularis*; 14. *L. juergensii*; 15. *B. obtusa*; 19. *A. subsessilis*; 20. *L. lingbyei*

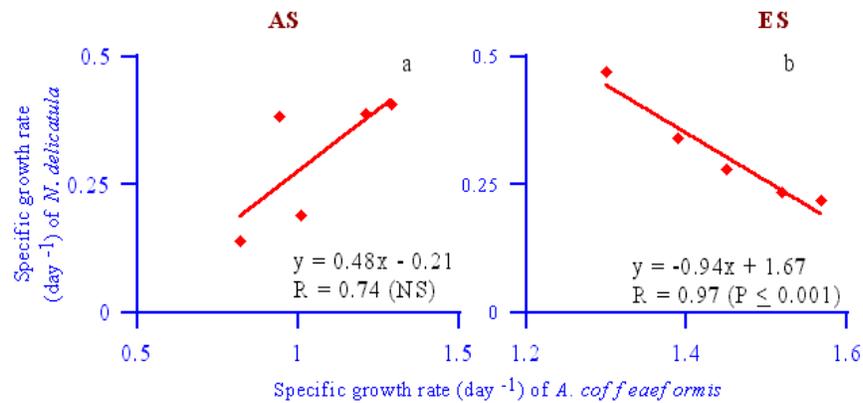


Fig. 4.6 Linear regression analysis of the specific growth rates of *A. coffeaeformis* and *N. delicatula* in AS and ES
NS=not significant

days old community was $\sim 0.2:0.85$ and linear regression analysis revealed a negative correlation between their growth rates with longer incubation period (Fig. 4.6). *A. coffeaeformis* occupied lesser area as compared to *N. delicatula* and even though the carrying capacity was not exceeded, *N. delicatula* cells were lost from the slide. Polynomial regression analysis between the percent coverage by the periphytic diatom community and species diversity in AS resulted in a trough, revealing a lower coverage at intermediate species diversity and an increase towards higher and lower species diversity (Fig. 4.7a) whereas in case of ES, the percent coverage and species diversity did not show any significant relationship (Fig. 4.7b).

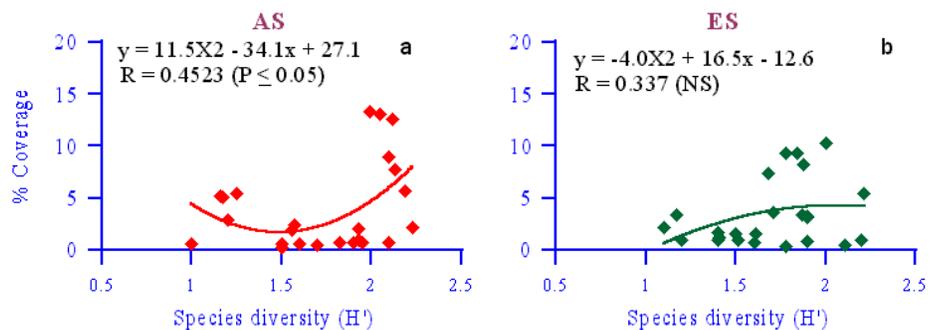
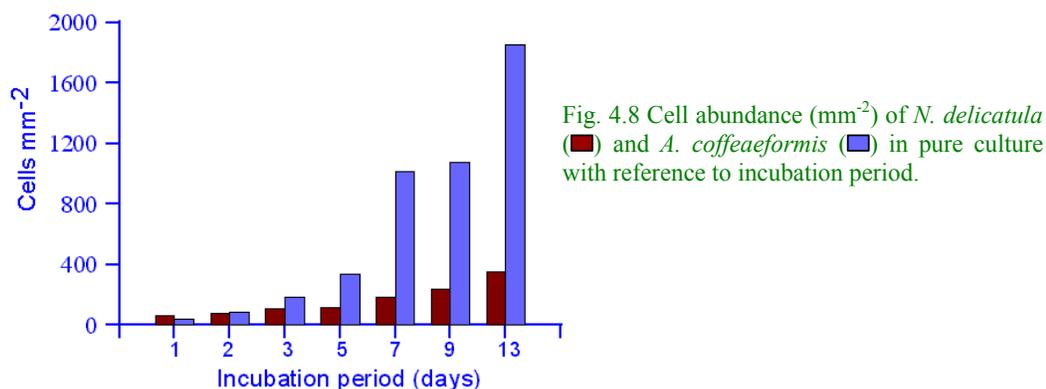


Fig. 4.7 Polynomial regression analysis between the periphytic diatom species diversity and percent coverage in AS and ES of case I
NS=not significant

Competition between *N. delicatula* and *A. coffeaeformis* in culture

Pure culture of *A. coffeaeformis* exhibited higher cell abundance as compared to *N. delicatula* with increase in incubation period (Fig. 4.8). The behavior of mixed diatom



culture of *A. coffeaeformis* and *N. delicatula*, when grown at various initial density ratios is given in Fig. 4.9. At an initial density ratio of *N. delicatula* and *A. coffeaeformis* ranging from 99:1 to 1:99, *A. coffeaeformis* showed an increasing trend

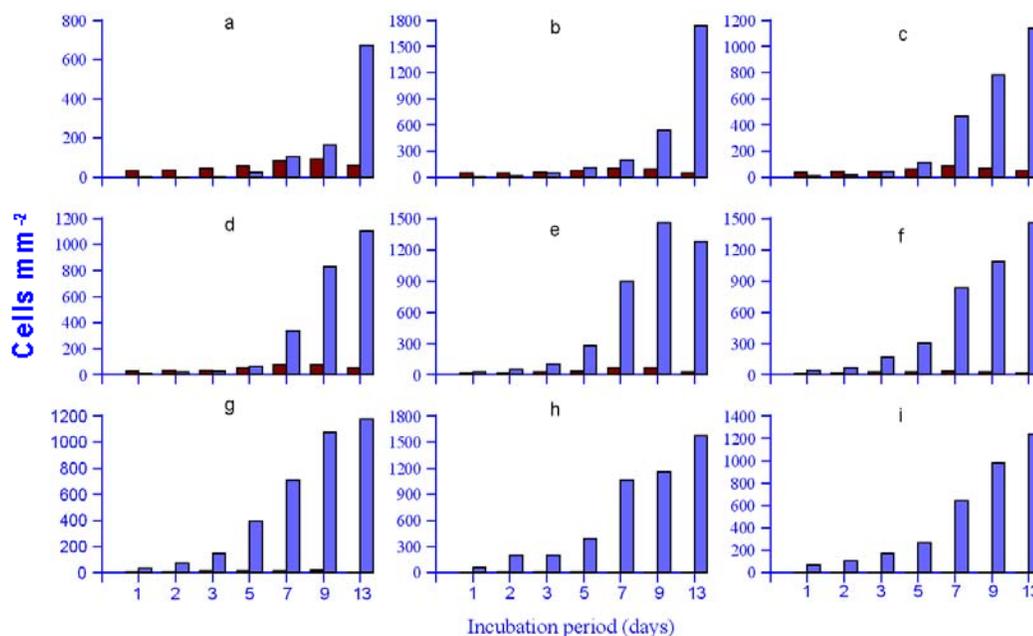


Fig. 4.9 Cell abundance (mm^{-2}) in mixed culture at different initial cell density ratios of *N. delicatula* (■) and *A. coffeaeformis* (■) (a) 99:1 (b) 95:5 (c) 85:15 (d) 75:25 (e) 50:50 (f) 25:75 (g) 15:85 (h) 5:95 and (i) 1:99.

with increase in incubation period (Fig. 4.9a-i). With increase in initial density from 1 to 50% of *A. coffeaeformis*, the time required to overtake *N. delicatula* reduced from 7 to 2 days (Fig. 4.9a-e). At densities ranging from 75% to 99%, the initial inoculum density of *A. coffeaeformis* being higher, dominated over *N. delicatula* throughout the incubation period (Fig. 4.9).

Case II (Inter and Intrageneric competition)

The periphytic diatom community from the environment differed substantially with exposure duration (Fig. 4.4, 4.10a, c, e & g, Appendix 4.3a-d, 4.4a-d, 4.12a & g, 4.13a & g). The dominant species were *N. delicatula*, *Navicula subinflata*, *Amphora turgida*, *Amphora hyalina*, *A. coffeaeformis* and *Biddulphia rhombus*. The changes in the community structure after incubation is shown in figures 10a, b, e and f and Appendix 4.3e-h in case of AS and figures 10c, d, g and h and Appendix 4.4e-h in case of ES. Incubation resulted in continued dominance of *N. delicatula* and *A. hyalina* irrespective of nutrient enrichment (e.g. the one day old community). Forms such as *B. rhombus* and *N. subinflata*, which also had a higher initial field inoculum, could not hold on to the substrata and were found in the sloughed off community (Appendix 4.3i-l and 4.4i-l). While *B. rhombus* was lost soon after incubation, high numbers of *N. subinflata* were encountered with longer incubation period. *N. delicatula* and *A. hyalina* were in less numbers in the sloughed off community. Incubation of the three days old community revealed that higher initial field inoculum of *A. turgida* resulted in its higher growth rate than *A. hyalina*, in ES (Appendix 4.4g, 4.13g-l). Similar pattern was observed in AS (Appendix 4.3g, 4.13a-f), even though the initial inoculum of *A. turgida* was low as compared to *A. hyalina*. In the four days

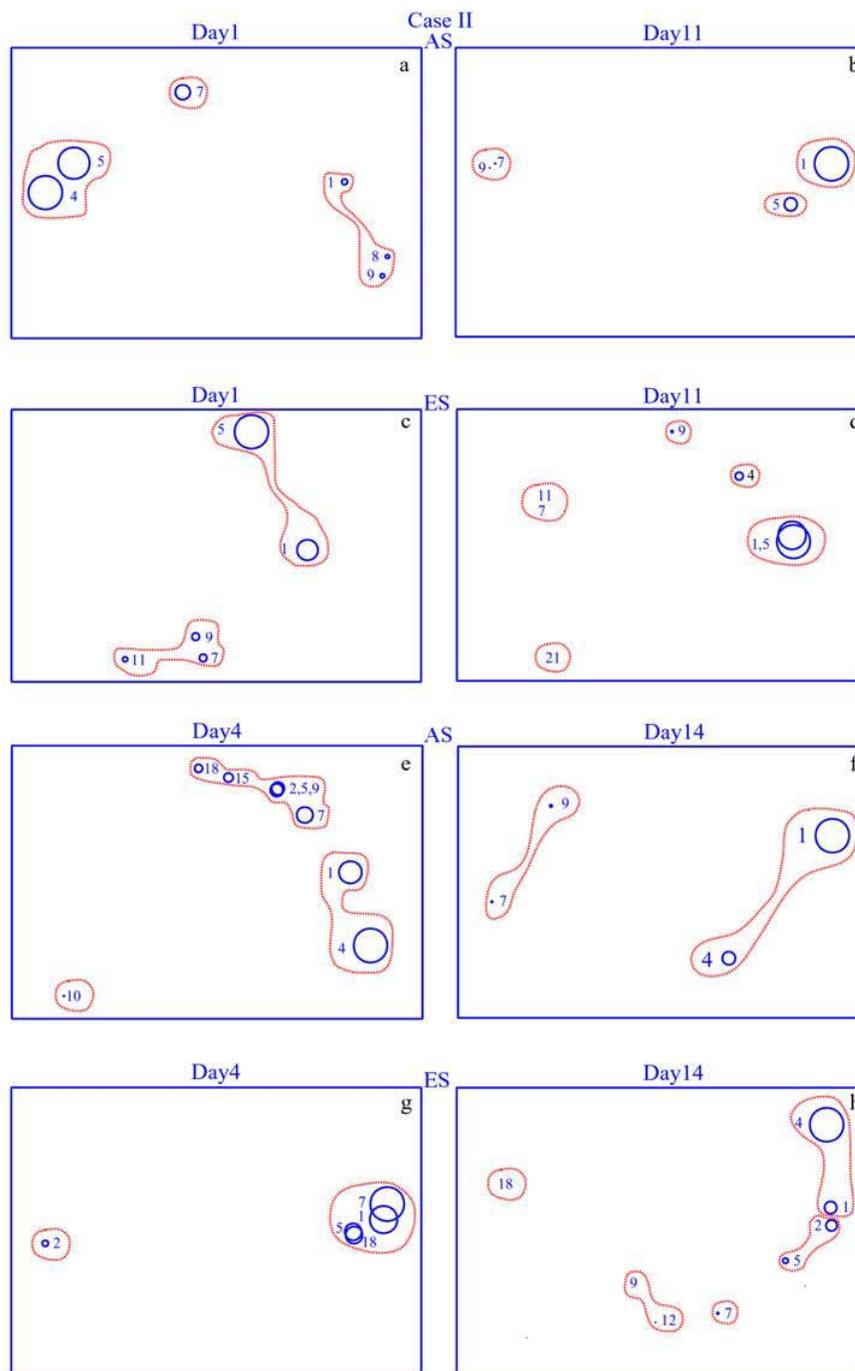


Fig. 4.10. Multidimensional scaling (MDS) ordinations for the case II periphytic diatom community incubated in AS and ES based on Bray-Curtis similarities. In the bubble plot, increasing size of circles indicate increasing abundance of a species (Stress = 0). Hatched lines indicate groups of species. 4.10b. *N. delicatula* (1) is the dominant diatom. 4.10a. *N. delicatula* (1), *P. angulatum* (8) and *N. subinflata* (9) are the dominant diatoms. 4.10c. *N. delicatula* (1) and *A. hyalina* (5) are the dominant diatoms. 4.10d. *N. delicatula* (1) and *A. hyalina* (5) exhibiting similar distribution. 4.10e, f and h. *N. delicatula* (1) and *A. turgida* (4) are the dominant diatoms. 4.10g. *N. delicatula* (1), *A. hyalina* (5), *B. rhombus* (7) and *G. marina* (18) are the dominant diatoms. 1. *N. delicatula*; 2. *A. coffeaeformis*; 4. *A. turgida*; 5. *A. hyalina*; 7. *B. rhombus*; 8. *P. angulatum*; 9. *N. subinflata*; 11. *C. marginatus*; 12. *C. scutellum*; 15. *B. obtusa*; 18. *G. marina*; 21. *A. longipes*

old community, introduction of *A. coffeaeformis* in ES was followed by an intrageneric competition between the three species of *Amphora* in ES (Appendix 4.4h, 4.14e-h). *A. turgida* turned out to be a comparatively stronger competitor (Fig. 4.10h). Simultaneously, an intergeneric competition was observed between the three species of *Amphora* and *N. delicatula*. In this competition, *A. turgida* and *N. delicatula* were found to be dominant. Comparatively, incubation of the four days old

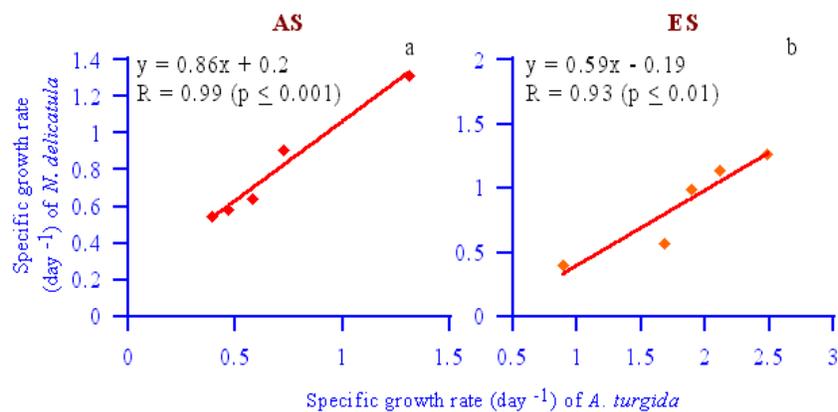


Fig. 4.11 Linear regression analysis of the specific growth rates of *A. turgida* and *N. delicatula* in AS and ES
NS=not significant

field community in AS, showed *A. turgida* and *N. delicatula* to be dominant (Fig. 4.10f, Appendix 4.3h, 4.14a-d). Linear regression analysis revealed a positive correlation between their growth rates with longer incubation period (Fig. 4.11a and 4.11b). The other species of *Amphora*, viz. *A. hyalina* and *A. coffeaeformis*, were observed in the sloughed off community along with *N. subinflata* and *B. rhombus*, which had a higher initial field inoculum (Appendix 4.3i-l). As in case I, it is observed that the area coverage of *A. turgida* was less and did not exceed the carrying capacity, and yet resulted in the loss of competitor species. Polynomial regression analysis revealed that the percent coverage by the periphytic diatom community decreased as the species diversity increased in AS (Fig. 4.12a) whereas in case of ES, species diversity and area coverage did not show any significant relationship (Fig. 4.12b).

Case III (Competitive exclusion or co-existence)

Dominance of periphytic diatoms in the differently aged slides differed (Fig. 4.4, Appendix 4.5a-d and 4.6a-d). In addition to *N. delicatula*, *N. subinflata*, *N. closterium*, *A. turgida*, *A. coffeaeformis* and *A. hyalina*, encountered in case I and II, this community included other major forms such as *Thalassionema nitzschioides* and *Nitzschia longissima*. The incubation of one-day-old field community showed *N.*

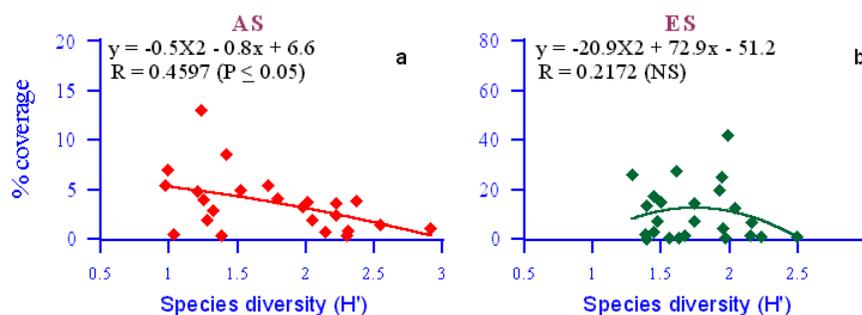


Fig. 4.12 Polynomial regression analysis between the periphytic diatom species diversity and percent coverage in AS and ES of case II
NS=not significant

delicatula and *N. subinflata* to continue their dominance irrespective of nutrient enrichment (Fig. 4.13a, b, c and d, Appendix 4.5e, 4.6e, 4.15a-h). In addition to these forms, the ES incubated community showed the presence of *N. closterium* whereas the AS incubated community showed the presence of *A. coffeaeformis*. Subsequent to incubation, the two days old-field community did not differ much with respect to nutrient enrichment (Appendix 4.5f, 4.6f, 4.16a-l). It comprised major forms of one-day field community and an addition of *A. turgida*. The initial inoculum of the *Amphora* species was low as compared to others. In case of the three days old community incubation in ES, *N. delicatula* was closely followed by *N. subinflata*, *N. longissima* and *A. coffeaeformis* (Appendix 4.6g, 4.17g-l). *N. closterium* and *T. frauenfeldii* with a high initial field inoculum were not found to establish on the original substrata and were found in the sloughed off community (Appendix 4.6i-l). The AS incubated three days old-field community had a high initial inoculum of *N.*

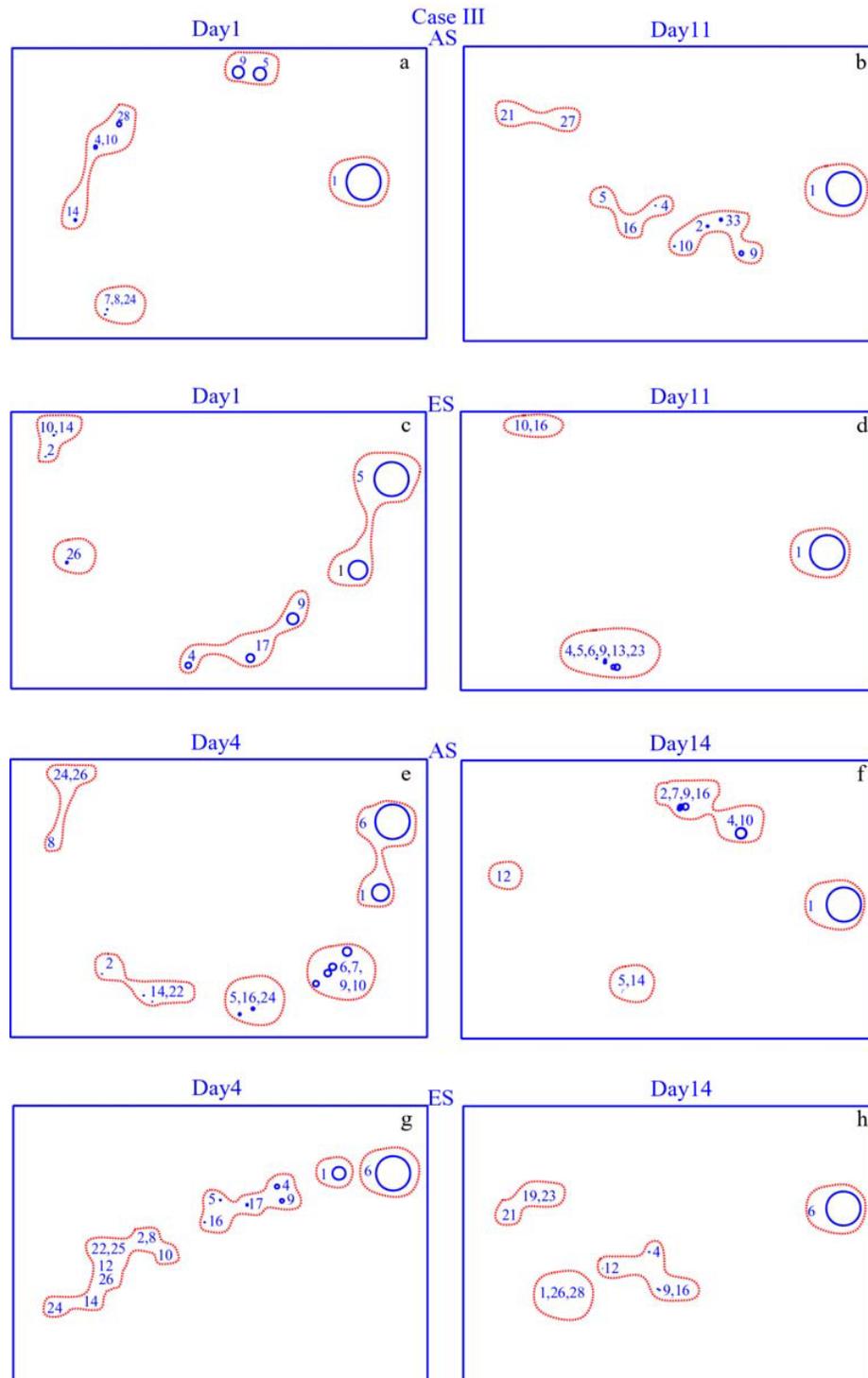


Fig. 4.13. Multidimensional scaling (MDS) ordinations for the case III periphytic diatom community incubated in AS and ES based on Bray-Curtis similarities. In the bubble plot, increasing size of circles indicate increasing abundance of a species (Stress = 0). Hatched lines indicate groups of species. 4.13a, b, d, h. *N. delicatula* (1) is the dominant diatom. 4.13f. Co-existence of *N. delicatula* (1), *N. closterium* (10) *A. turgida* (4) and *A. rostrata* (16). 4.13e. *N. delicatula* (1) and *N. longissima* (6) are the dominant diatoms. 4.13c. *N. delicatula* (1) and *A. hyalina* (5) are the dominant diatoms. 4.13g, h. *N. longissima* (6) is the most dominant diatom 1. *N. delicatula*; 4. *A. turgida*; 5. *A. hyalina*; 6. *N. longissima*; 7. *B. rhombus*; 8. *P. angulatum*; 9. *N. subinflata*; 10. *N. closterium*; 12. *C. scutellum*; 13. *N. angularis*; 14. *L. juergensii*; 16. *A. rostrata*; 17. *T. nitzschioides*; 19. *A. subsessilis*; 21. *A. longipes*; 22. *L. paradoxa*; 23. *P. seriata*; 24. *T. eccentrica*; 25. *N. septentrionalis*; 26. *M. nummuloides*; 27. *G. serpentina*; 28. *M. membranacea*; 33. *N. sigma*

delicatula, *N. subinflata*, *T. nitzschioides*, *N. longissima* and *N. closterium* (Appendix 4.5g, 4.17a-f). However, out of these only *N. delicatula* and *N. subinflata* continued their dominance after incubation. Rest of the forms were found in the sloughed off

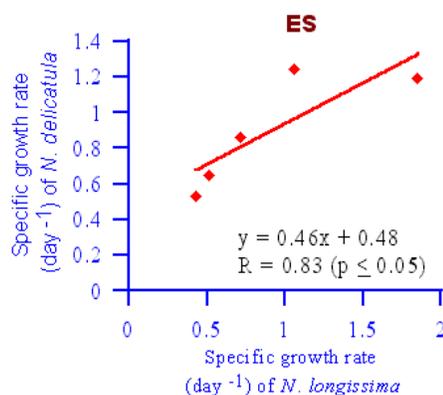


Fig. 4.14 Linear regression analysis of the specific growth rates of *N. longissima* and *N. delicatula* in ES
NS=not significant

community (Appendix 4.5i-l). *N. longissima* was a dominant component of the four days old field community (73%) and this species maintained its dominance over *N. delicatula*, in ES (Fig. 4.13h). Linear regression analysis revealed a negative correlation between their growth rates with longer incubation period (Fig. 4.14). In the sloughed off community, *N. delicatula* was seen, along with *N. subinflata* and *A. coffeaeformis*. Dominance of *N. longissima* was reflected in the area coverage wherein it exceeded the carrying capacity of the substrata. In case of AS, *N. longissima*, even though the initial inoculum was high, could not grow and gave way to other forms such as *N. delicatula*, *N. closterium*, *N. subinflata*, *A. turgida*, *A. hyalina* and *A. coffeaeformis* (Fig. 4.13f). In this case, difference between the original substrate (Appendix 4.5e-h) and sloughed off community was minimal (Appendix 4.5i-l). Polynomial regression analysis revealed that the percent coverage by the periphytic diatom community was higher at lower species diversity in ES (Fig. 4.15b) whereas in AS a trough was observed which showed lesser area coverage at intermediate species diversity (Fig. 4.15a). Area of the individual cell for each of the periphytic diatom species is given in Table 4.1.

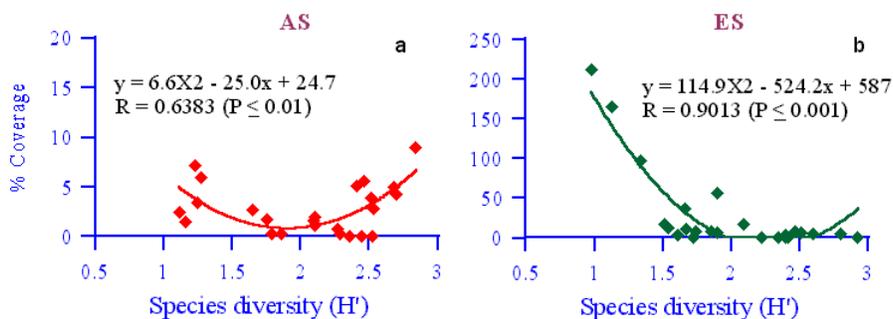


Fig. 4.15 Polynomial regression analysis between the periphytic diatom species diversity and percent coverage in AS and ES of case III
NS=not significant

4.4 DISCUSSION

An increase in periphytic diatom cell abundance in the field with longer submersion period reveals that the effect of cell immigration and cell reproduction overrides that of the other factors (Fig. 4.1a). Subsequent to incubation of the one to four days old-field diatom community in laboratory conditions, where physical disturbance and grazers were absent, increase in the periphytic diatom population, can be attributed to cell multiplication (Fig. 4.1b). However, these communities included a large number of rare species (Fig. 4.5, 4.10, and 4.13, Appendix 4.1e-h to 4.6e-h, Table 4.1), which did not contribute to the corresponding assemblage by building up a sizeable population.

In this study, three scenarios emerged from the interactions between forms of the periphytic diatom community (Fig. 4.16a-c). In case I (Fig. 4.16a), the major players, *A. coffeaeformis* and *N. delicatula* are the natives of the surrounding intertidal sand flat, where *A. coffeaeformis* is the abundant form (Mitbavkar and Anil 2002). However, *A. coffeaeformis* represents the ambient waters in low numbers, which limit their colonizing capabilities in the periphytic diatom community (e.g. substratum away from their natural habitat) in this area. Subsequent to incubation of the different

aged (one to four days old) field communities, the increasing trend of total diatom abundance observed for the one and two days old-field communities, being higher for

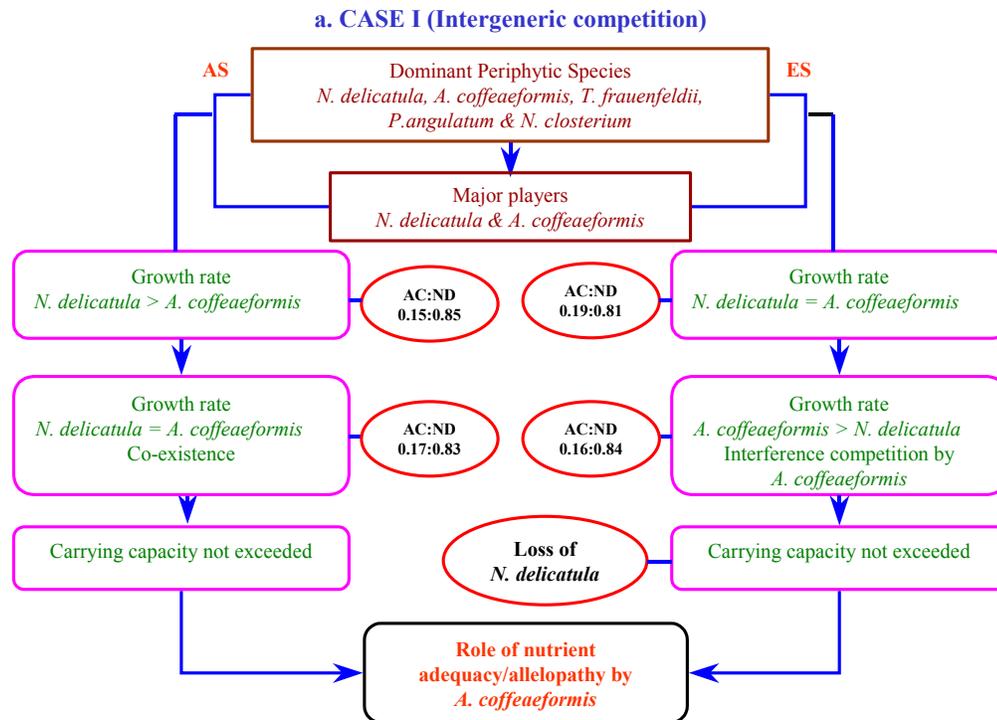


Fig. 4.16a Schematic representation of the periphytic diatom community interactions encountered during the laboratory incubations in case I

ES than AS, was reversed for the three and four days old-field communities. Reason for this reversal was the elimination of *N. delicatula* from the substrate by *A. coffeaeformis* in the presence of additional nutrients. *A. coffeaeformis* exhibited a faster growth rate and surpassed *N. delicatula* within four days of incubation, at an initial field inoculum ratio of 0.2:0.85. Though *A. coffeaeformis* was increasing in abundance thereby resulting in a higher composition, exceeding that of *N. delicatula*, it was not reflected in the area coverage, because of its smaller size compared to *N. delicatula* (Table 4.1). In this case, even though the substrate carrying capacity had not exceeded, cells of *N. delicatula* were being eliminated from the substratum. Conversely, both these species co-existed in AS. In a study carried out by Buzzelli et

al. (1997) on *A. coffeaeformis*, cultures showed the best growth rate at 900 μM nitrate and 36 μM orthophosphate, which is close to ES N: P ratios.

In another laboratory experiment carried out in order to study the competition between *A. coffeaeformis* and *N. delicatula* in ES, competitive influence of the population of *A. coffeaeformis* on *N. delicatula* was independent of the initial cell density ratio, wherein it could overtake its competitor species even at 1% initial inoculum. But, as the initial density increased the rate of competitive ability to overtake increased thus reducing the time, from 7 days in case of 1% to 2 days in case of 50%. This shows that even though the competitive abilities are independent of the initial density, it does have an influence over the time within which the superior competitor can overtake its counterpart. Growth limitation due to nutrient competition between two species may be explained by higher demands and higher uptake rates of nutrients by *A. coffeaeformis* when nutrients were available above a threshold level.

According to the classical ecological competition theory, the one species that can best utilize the limiting nutrients should displace all its other competitors, under idealized conditions. However, coexistence of many species in a natural ecosystem puts such a situation at bay. This discrepancy between the nature and theoretical prediction was referred to as the 'paradox of the plankton' by Hutchinson (1961). Temporal and spatial variability of nature (Hutchinson 1961; Richerson et al. 1970), mortality rates from differential grazing (Paine 1966; O' Brien 1974) and differences in the ability of species to acquire nutrients (Stewart and Lewin 1973; Greeney et al. 1973; Petersen 1975) are the theories put forth to explain such a situation. A study by Tilman (1976) has shown that long-term co-existence of competing species can occur only when the growth rate of each species is limited by a different nutrient. It has been suggested

that the co-existence may be the result of the infinite number of possible ratios of resource concentrations that can occur in evolving a natural system (Tilman 1982).

Probably such exclusion is also aided by allelopathy wherein members of one species interferes with or directly harm members of a second species, which ultimately loses the competitive battle through chemical interactions (Valiela 1984). Production of such a compound would be regulated by nutrients. Such interaction has also been reported among marine phytoplankton species and is suggested to play an important role in ecology. Pratt (1966) found a temporary inhibition of *Skeletonema costatum* by filtrates taken from a culture of *Olithodiscus luteus* only when the *Olithodiscus* concentration exceeded 108 cells liter⁻¹. *A. coffeaeformis*, which is known for domoic acid production, a water-soluble toxin (Maranda et al.1989), is reported to have certain toxic effects (Buzzelli et al. 1997). Since the toxicity of these allelopathic compounds depends on the amount released and on the abundance of both, *A. coffeaeformis* and *N. delicatula* (target species), this effect was not observed in the absence of additional nutrients. This possibility has to be investigated in detail.

It is evident from this observation that the species composition will determine the outcome of a particular community interaction. Grazing cannot influence community structure, since for such an effect; selective grazing of a particular key species is necessary. Such a process at the micro scale level has to be investigated in detail. Area coverage was controlled by species diversity, which was in turn dependent on the nutrient supply. In the presence of additional nutrients, the elimination of *N. delicatula* by *A. coffeaeformis*, resulted in a non-significant relationship between the species diversity and percent coverage (Fig. 4.7b). However, in case of AS, similar growth rates of both these forms resulted in a significant relationship between the species diversity and percent coverage (Fig. 4.7a).

As compared to case I, where only one species, *A. coffeaeformis*, competed with *N. delicatula* for the dominant position, in case II (Fig. 4.16b), the competition was tougher involving three species, *A. hyalina*, *A. turgida* and *A. coffeaeformis*, which competed with *N. delicatula*, thus playing an important role in community structure. In the presence of nutrients, an intrageneric competition was brought about between the three species of *Amphora*, with a simultaneous intergeneric competition with *N. delicatula*. Ultimately, *A. turgida* turned out to be a better competitor in the intrageneric competition. In the intergeneric competition, both *A. turgida* and *N. delicatula* were dominant. However, in the absence of additional nutrients, the other congeneric competitor species were eliminated from the substrata and *A. turgida* proved to be a superior competitor, along with *N. delicatula*. The losers were seen in the sloughed off community. The sloughed off community showed the presence of *N. subinflata* (one day old community; ES) and *B. rhombus* (three days old community; AS). These forms were not recorded on slide, which indicates that their presence went unobserved due to their low numbers. Also, an increase in cell numbers after an initial slow loss was observed which shows that these cells were present and were multiplying on the slide, however at a slower rate than the other major forms. *A. turgida* and *N. delicatula* could grow in both, nutrient enriched as well as in conditions where additional nutrients were absent.

From these observations, it is evident that all three members of *Amphora* probably have similar nutrient requirements. Even then they co-exist in ES, where the common nutrient supply is sufficient, whereas in AS, where the nutrients could be limiting, according to the resource competition theory, only one of the three species of *Amphora*, i.e., *A. turgida*, which could possibly sequester the limiting nutrients at a faster rate thus making it unavailable to the other species of *Amphora* i.e., *A. hyalina*

and *A. coffeaeformis*, proved to be a successful competitor. This species was found to co-exist with *N. delicatula*, which could be due to a difference in nutrient requirements. Competition for nutrients has been reported by Mickelson (1979), wherein *Thalassiosira gravida* is rapidly displaced from ammonium limited chemostats by *Skeletonema costatum* and *Chaetoceros septentrionalis*.

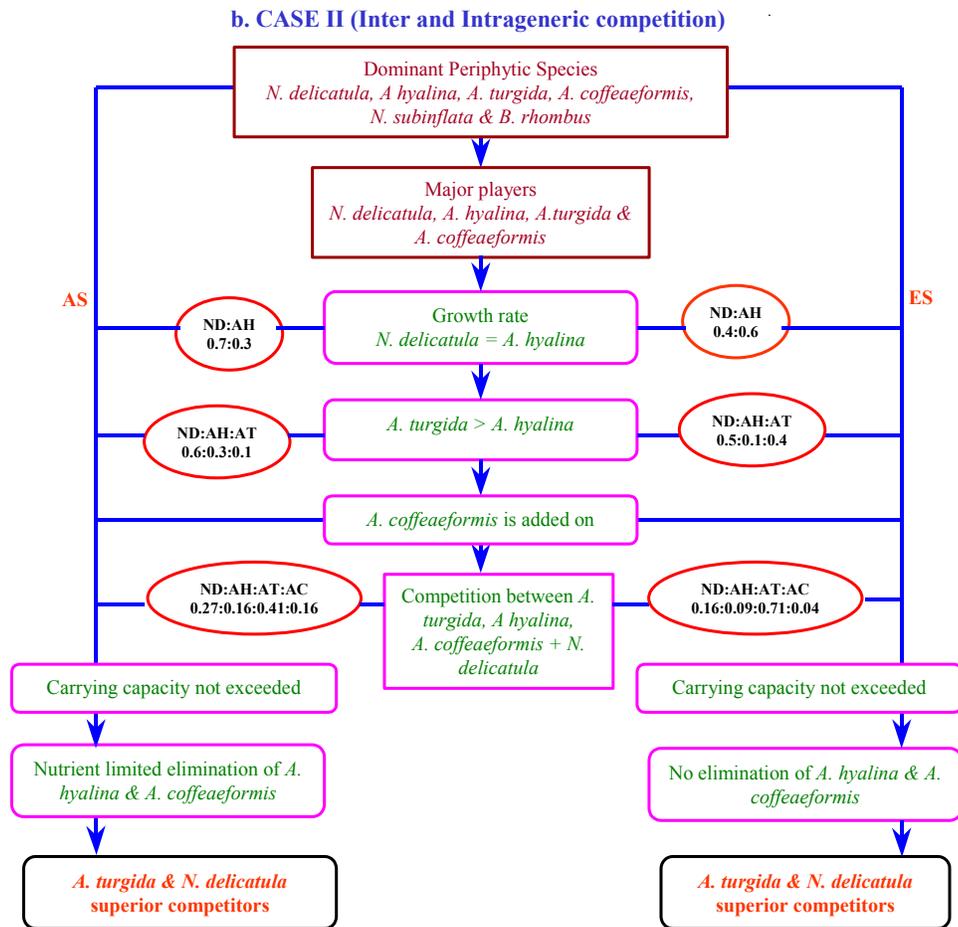


Fig. 4.16b Schematic representation of the periphytic diatom community interactions encountered during the laboratory incubations in case II

A. coffeaeformis, the key player of case I was not given an opportunity to exert its influence in this interaction, as it could not reach the optimal density for bringing about the competitive effect. Even though *A. turgida* played an important role in structuring the diatom community, it did not contribute much to the carrying capacity of the substrata. In case of AS, lowered species diversity due to the elimination of *A.*

hyalina and *A. coffeaeformis*, resulted in a significant relationship between the species diversity and percent coverage, with lowered diversity favoring higher area coverage (Fig. 4.12a).

In case III (Fig. 4.16c), *N. longissima* was the key controller of the community structure. In the presence of additional nutrients, substrate surface area available for attachment became increasingly important for *N. longissima*, thereby resulting in a direct interference competition for space. The probable allelopathic effect shown by *A. coffeaeformis* in case I was not experienced in the present scenario, wherein its initial field inoculum was low and it was displaced by *N. longissima* before it reached the appropriate numbers. *N. delicatula*, *N. subinflata* and *N. closterium* were likewise completely eliminated from the shared environment through competitive exclusion. These forms were seen in the sloughed off community. Such, competitive exclusion has been reported by Hambright and Zohary (2000) in their study in a subtropical area, where seasonal dominance of microcystis (blue-green algae) eliminates other species, light being the primary limiting resource. Sommer (1984) through multispecies competition experiments demonstrated that co-existence could occur when a key nutrient is supplied at regular intervals. The high densities achieved by the opportunistic species, *N. longissima* diminished the carrying capacity of the substrate and probably denied access to the key nutrients to its competitors, thereby suppressing their growth and resulting in lowered diversity. In spite of the lowered species diversity, the larger size of *N. longissima* (Table 4.1) resulted in higher percent coverage. This is evident from the significant relationship between species diversity and percent coverage, with the lowered diversity favoring higher percent coverage (Fig. 4.15b). In the absence of additional nutrients, *N. longissima* could not prosper, even though its initial field inoculum was high, probably because it could not

sequester the key nutrients present in low concentrations. This gave way to forms such as *N. subinflata*, *N. closterium* and *A. turgida* which could grow efficiently even

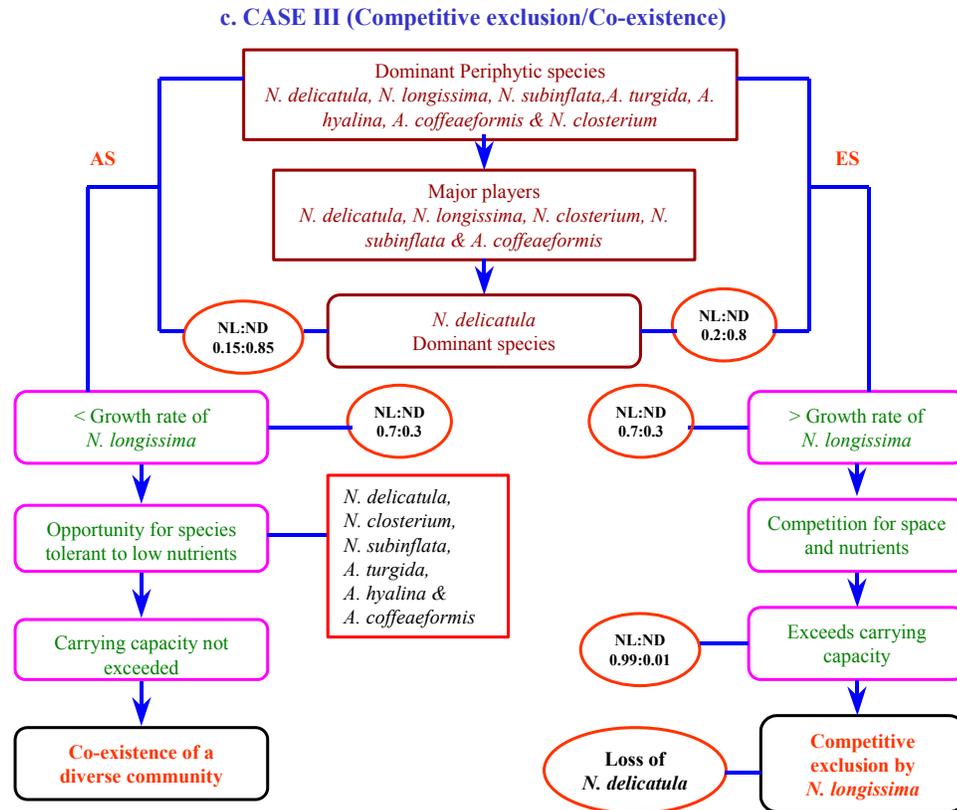


Fig. 4.16c Schematic representation of the periphytic diatom community interactions encountered during the laboratory incubations in case III

in the absence of additional nutrients, each probably having a different resource requirement, thereby resulting in a diverse community. Here, increased species diversity resulted in higher percent coverage (Fig. 4.15a). Another reason for the differences in community structure between AS and ES could be the half-saturation constant which is usually lower for the small-sized organisms as compared to the larger ones. Thus, in oligotrophic conditions small phytoplankton thrive better than larger ones. Shifts in diatom community structure have been reported by Chiba and Saino (2002) in the offshore areas of Japan Sea, wherein low concentrations of phosphate caused a change in the dominant diatom species from those adapted to high nutrient conditions to those adapted to low nutrient conditions.

Hillebrand and Sommer (2000) demonstrated that enhanced nutrient supply decreased micro algal diversity with increase in colonization time (12 weeks). Our study which was restricted to 4 days due to the appearance of macro organisms, revealed an increasing trend in species diversity. This difference could be due to the spatial variations. Subsequent incubation of these communities in controlled conditions revealed differences in species diversity depending on nutrient availability. While in ES, the species diversity decreased in case I and III and remained stable in case II; in AS, species diversity remained stable in case I and III and decreased in case II. Species interactions in case I (intergeneric competition), case II (inter and intrageneric competition) and case III (competitive exclusion and co-existence) might have resulted in the variations in species diversity (Fig. 4.4).

Since every substrate will have a particular carrying capacity, each of the species belonging to the diverse community will try its best, to own a territory for itself. The carrying capacity is mainly determined by the substrate area coverage by a particular species. However, a species might be increasing in abundance but the area covered will be less due its smaller size, as seen in case I and II (Fig. 4.16a and b). In such situations, faster uptake of nutrients or chemical interactions (allelopathy) between competing species is one of the possibilities to own space (case I). In another scenario of intrageneric competition, wherein members of the same genus might have similar resource requirements, possibility to own space is by displacing the competitors via resource competition (case II). The superior competitor utilizes nutrients at a faster rate thereby making it unavailable to its competitors. On the other hand, if a species is larger in size it can occupy space through physical exclusion (competitive exclusion) of the competitor species in the presence of sufficient nutrients (case III) (Fig. 4.16c). However, competitive trait of a particular species clicks on at an appropriate cell

density ratio of the competitive and target species. Nutrient adequacy is another factor, which can trigger the competitive expression.

Chapter 5

Morphological changes and detection of diatoms

5A. Study of life cycle of diatoms

5A.1 Introduction

Diatom life cycle includes vegetative and sexual stages. They also, but may not necessarily pass through a seed-like phase known as the resting stage. Resting stages are of two different types, resting spores and resting cells. While, the resting spores are heavy-walled stages that are morphologically distinct from vegetative cells, the resting cells are similar to vegetative cells, except for altered cytoplasmic characteristics (Anderson 1975; Hargraves and French 1975; Hoban et al. 1980; Sicko-Goad et al. 1986; Round et al. 1990). Normally, diatoms reproduce by vegetative division and for many species the vegetative stage is the only one commonly observed (Garrison 1984). According to the Mc Donald – Pfitzer hypothesis most diatoms decrease in valve diameter during the vegetative phase of the life cycle (Round 1972). This phenomenon is unique to diatoms and can be attributed to frustule structure and morphogenesis. During this “shrinking division” mode of asexual reproduction, the two valves of the test separate. Each forms the epivalve of a daughter cell, and a new hypovalve is secreted within each of the parent valves. The result is one cell that is the same size as the parent cell, and one cell that is slightly smaller. Due to the rigidity of the test material, growth of the cell is impossible once the test is secreted. Thus, the average diatom size gets progressively smaller with each round of replication. Sexuality or some similar size regenerative process is thus periodically necessary to keep a population from passing the lower limit of size viability (French and Hargraves 1985). The restoration of cell size by sexual reproduction is a unique feature of diatoms (Drebes 1977). The sexual reproduction mode allows for growth of the zygote to relatively large size. It is the escape hatch for diatoms from the ever-shrinking asexual mode (Drebes 1977; Round et al. 1990;

Mann and Stickle 1993). An alteration of physical or chemical factors has typically been used to initiate auxospore formation, including changes in light intensity, photoperiod, temperature and salinity (Drebes 1977).

In this study, an effort was made to observe the different stages in the life cycle of two pennate diatoms, *Amphora coffeaeformis* and *Navicula delicatula* by exposing them to different salinities. The study focused on these two species, as they are the major members representing the microphytobenthic community of intertidal sediments.

5A.2 Materials and Methods

5A.2.1 Isolation and maintenance of cultures

The two species of pennate diatoms, *A. coffeaeformis* and *N. delicatula* were isolated from the intertidal sediments of the Dias Beach, near Dona Paula Bay, west coast of India (15° 27' N 73° 48' E). Cultures of these two species were maintained in the exponential growth phase by weekly reinoculation into fresh culture medium, prepared with autoclaved filtered 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 12 h light: 12 h dark photocycle.

5A.2.2 Experimental protocol

The culture medium f/2 was prepared in filtered autoclaved seawater with salinities ranging from 5 to 40 psu. The culture medium with each of these salinities (5 ml) was transferred to polystyrene multiwells (Corning 430343; in triplicates). To these multiwells, cells of *A. coffeaeformis* and *N. delicatula* were inoculated at an initial density of 1000 cells ml⁻¹ from an exponentially growing parent culture. These wells were examined everyday to observe any morphological changes that the cells

undergo. Cultures were observed by inverted microscopy (Olympus IX71) and photomicrographs were taken using digital camera (Kodak DC 290).

5A.3 Results

Vegetative or asexual mode of reproduction by cell fission was observed as the predominant method of reproduction in both the species of diatoms at all the

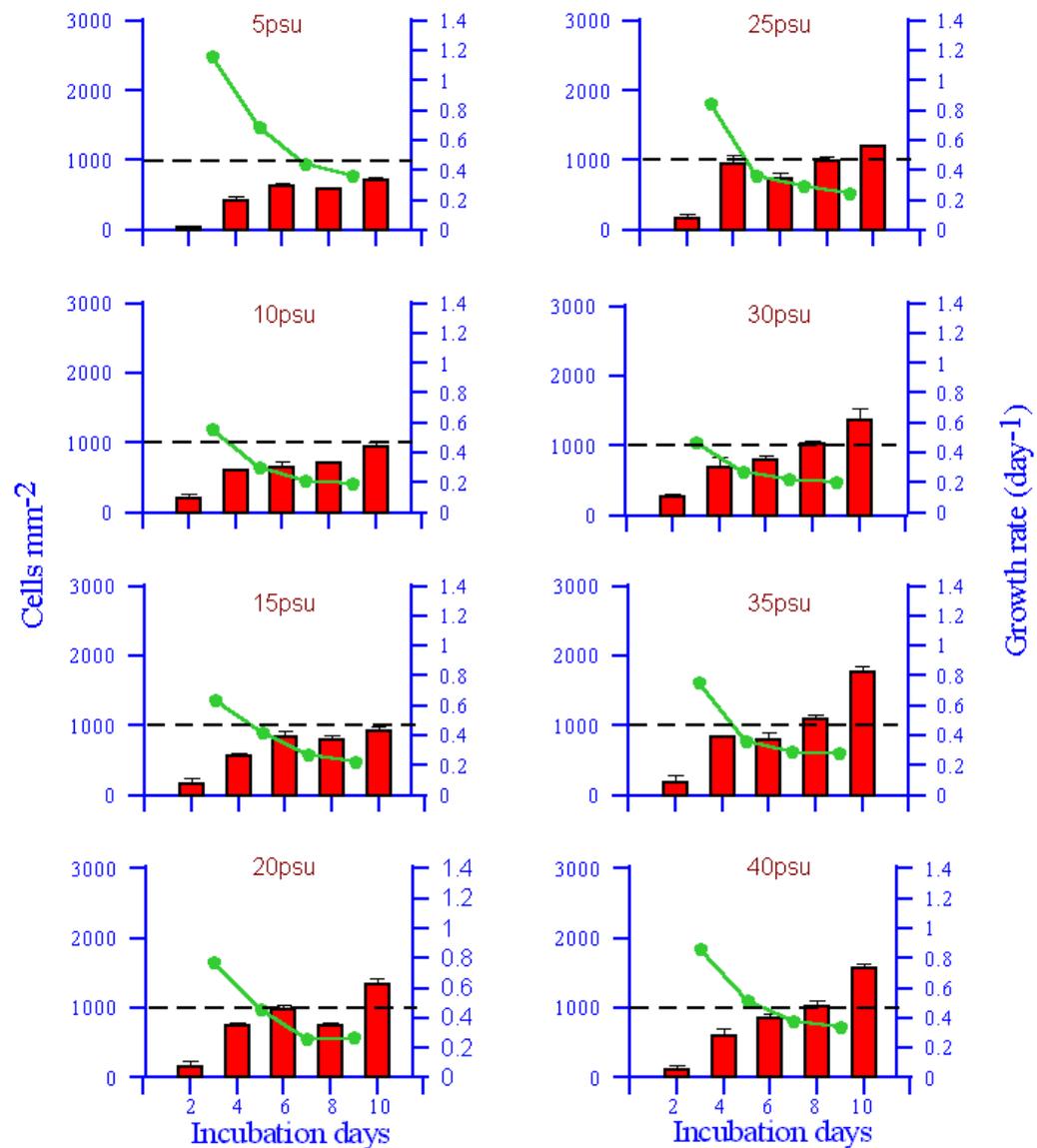


Fig. 5A.1 Cell abundance and specific growth rate of *Navicula delicatula* at different salinities. (Bars indicate cell abundance and line graph indicates specific growth rate)

experimental salinities. Cell abundance and growth rate of the two species at different salinities is given in Fig. 5A.1 and 5A.2. Both the species exhibited faster growth at higher salinities i.e., 30 to 40 psu compared to lower salinities.

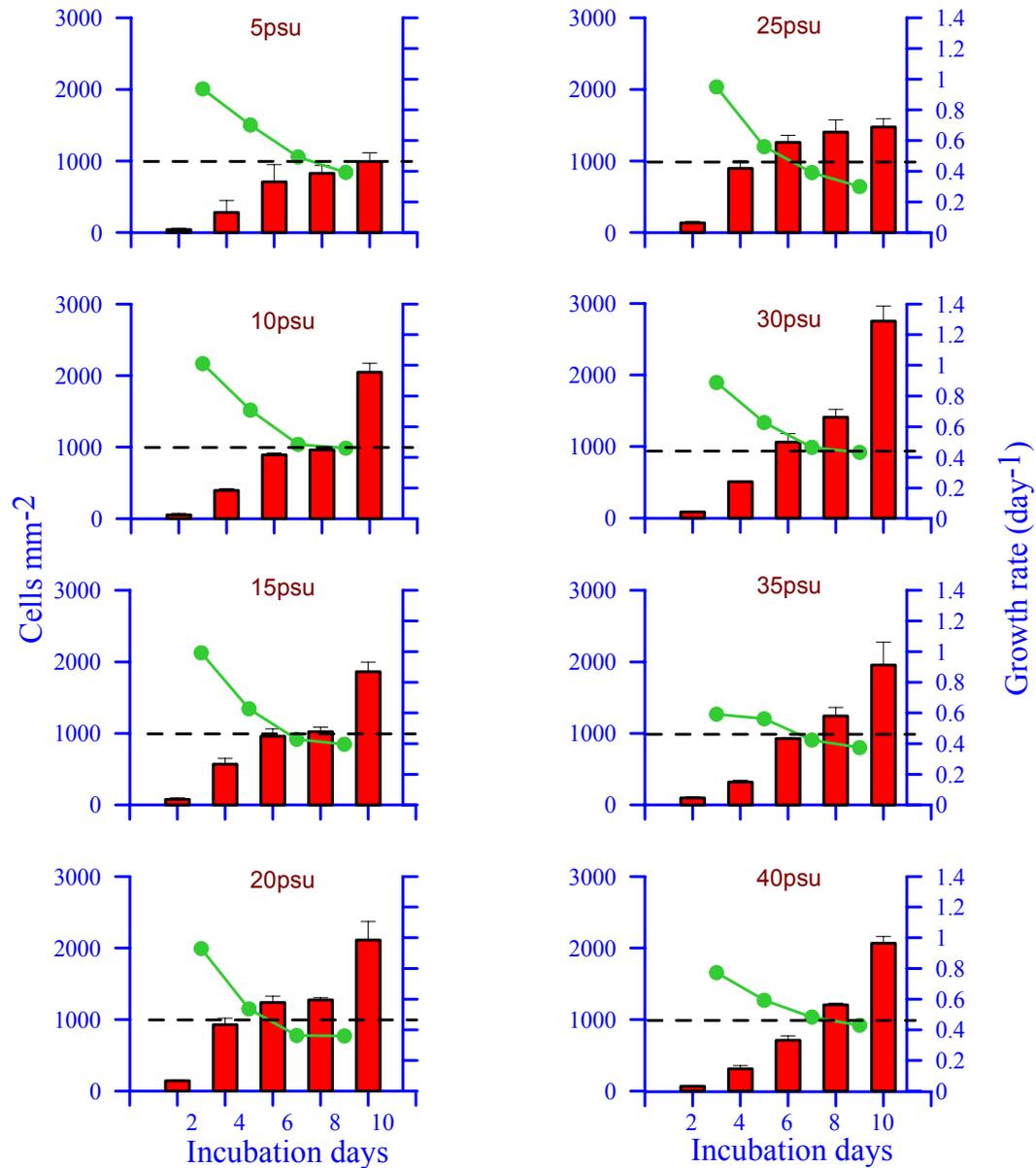


Fig. 5A.2 Cell abundance and specific growth rate of *Amphora coffeaformis* at different salinities. (Bars indicate cell abundance and line graph indicates specific growth rate)

In both species, sexual reproduction occurred in low numbers as part of a normal life cycle process at all salinities exposed. However, in case of *N. delicatula* an abrupt change in salinity i.e., when parent clones growing at 35 psu were mixed directly into a medium of 5 psu and 10 psu, induced sexual reproduction. Hence, at lower salinities the growth rate of *N. delicatula* was slower than that of *A. coffeaeformis*.

Sexual reproduction showed the following steps in *N. delicatula* (Plate 5A.1a) and *A. coffeaeformis* (Plate 5A.1b).

First stage: Pairing of parent cells (gametangiogamy) wherein gametangia contacted each other valve-to-valve, lying parallel.

Second stage: Gametogenesis wherein each cell in a pair divides meiotically forming spherical gametes. Each gametangium produces two morphologically isogamous nonflagellated gametes.

Third stage: The frustules opened completely, permitting both gametes in one cell to move by amoeboid action toward the passive gametes in other gametangium.

Fourth stage: Plasmogamy wherein only one pair of gametes fused at a time to form a spherical zygote.

Fifth stage: Resulting zygote then started to expand to form auxospore although not synchronously. Sometimes one auxospore enlarged relatively rapidly to its maximal size, whereas the other was still only at the beginning of expansion. The two auxospores eventually attained a similar length. Most of the auxospore volume appeared to be occupied by a single vacuole.

Sixth stage: The restored initial cell was formed inside the auxospore.

5A.4 Discussion

Phytoplankton life cycle stages are of importance in taxonomy and systematics. The present study shows that low salinity stress induces sexual reproduction in the pennate diatom, *N. delicatula* but not in *A. coffeaeformis*.

Both the species acquired greater cell density at 30 to 40 psu but could successfully maintain themselves even at lower salinities (5-25 psu). Low salinity may be one of any number of triggers or stresses that induce an asexual population to resort to sexual reproduction as in case of *N. delicatula*. Comparatively, *A. coffeaeformis* was found to be better tolerant to lower salinities. This showed that this species is euryhaline capable of tolerating a wide range of salinities ranging from 5 psu to 40 psu. While *N. delicatula* is comparatively less tolerant to lower salinities (5-10 psu) wherein there is a reduction in the normal vegetative division with increase in sexual reproduction through auxospore formation. This reveals that sexual reproduction, other than a mode of regaining normal cell size, can be induced on sudden exposure to stress conditions such as salinity variations.

Ragothaman and Rao (1978) have reported auxospore formation in *A. coffeaeformis* at lower salinities. However, in their study any increase in cell length was assumed to have been derived by auxospore formation and there was no visual observation of the actual auxospores and the process of sexual reproduction. Although resting stages were not observed for either of the two species during this study, Anderson (1975; 1976) has reported resting cell formation in *A. coffeaeformis*, when placed in continuous dark. No such reports are available in case of *N. delicatula*.

Howell and Pfister (1983) have studied the sexual life history of *Amphora veneta* from the natural population. However, much of their work focuses on the

morphological and ultrastructural features of the mature initial cell. In case of *Navicula*, sexual reproduction has been studied in *Navicula cuspidata* (Cohn et al. 1984; Schoeller et al. 1984), *Navicula directa* (Mizuno 2000) and *Navicula oblonga* (Mann and Stickle 1989). As observed for other pennate diatoms (Mann 1982; Chepurnov and Mann 1997; Davidowich and Bates 1998), the gametes of *A. coffeaeformis* and *N. delicatula* observed were morphologically indistinguishable from each other and had no flagella.

The number of diatom species in which sexual reproduction has been observed is very small relative to the total number of species recognized (Round et al. 1990). This is due to the difficulty of observing sexually reproducing cells in the natural environment. First and foremost, the ratio of auxospores to vegetative cells is low because relatively few individuals may be involved in sexual reproduction at any one time (Jewson 1992). Also the association between pairs of gametangia is likely to be so weak as to be disturbed during normal sampling (Davidowich and Bates 1998). However, this study will help and simplify identifying their different stages of life cycle in nature.

5B. Effects of low temperature on diatoms

5B.1 Introduction

Freezing is lethal to most living systems, yet it can also preserve cells and their constituents. It slows or stops some biochemical reactions, but it accelerates others (Mazur 1970). At temperatures below -150°C , biophysical processes do not affect organisms (Mazur 1964), and at the temperature of liquid nitrogen, temperature variability is independent of storage time (Morris 1981). By this means, the symptoms of aging such as reduced morphological competence and secondary product formation are avoided.

Cryopreservation has been widely used in medical and veterinary sciences, but its application in marine science is new and is only now being explored as an innovative adjunct in biotechnology. Cryopreservation studies include storage of spermatozoa of invertebrates and fish (Dunn and McLachlan 1973; Iwata et al. 1989; Kurokura et al. 1986; Kurokura et al. 1989), cryopreservation of rotifer (*Brachionus plicatilis*) (Toledo and Kurokura 1990), bivalve embryos (Renard 1991; Renard and Cochard 1989) and barnacle larvae (*Balanus amphitrite*) (Anil et al. 1997). Cryopreservation is an especially useful technique for the maintenance of phytoplankton culture collections (McLellan et al. 1991). Relatively few investigations have dealt with the effect of cryopreservation on algal cells (Hwang and Horneland 1965; Morris 1976a, b; McGrath and Daggett 1977) and more particularly on marine ones (Tsuru 1973; Saks 1978; Ben Amotz and Gilboa 1980; Meyer 1985; Fenwick and Day 1992; Levy and Zamir 1994; Bertrand et al. 1996). When algal cultures are maintained at an actively growing state, there is always the possibility of contamination or of genetic changes causing a gradual change in the hereditary makeup of the organism. For

these reasons, as well as because it offers a great saving in time, effort, and space, algal cultures are subjected to long-term preservation.

It is well known that the use of cryoprotectants is important to prevent cell damage during freezing. The absence of an effective cryoprotectant leads to the formation of intracellular ice crystals, which puncture cell organelles and cell membranes. Also, water within the medium freezes, cells become dehydrated, altering the salt concentration and causing morphological or biological changes after recovery. Grout and Morris (1987) reviewed the adverse effects of freezing on cells. They included, osmotic stress induced by freezing out of water. During formation of the ice lattice, dissolved solutes become highly concentrated (Pitt 1990), producing a hyperosmotic environment, which may lead to cell death. The present work evaluates the tolerance of two co-existing benthic diatom species, *Amphora coffeaeformis* and *Navicula delicatula* to low temperature and its effect on their internal morphology and viability. These two are the most abundant diatom species encountered in the intertidal sediments of the Dona Paula Bay (Mitbavkar and Anil 2002). Of these two species, *N. delicatula* also dominates the ambient waters and the microfilm population (Smita and Anil 2000). *A. coffeaeformis* is a species widely studied for its pioneer role in biofouling (Cooksey 1981; Cooksey and Cooksey 1980, 1984, 1986).

5B.2 Materials and Methods

5B.2.1 Culture

Flasks containing 100 ml of f/2 media (Guillard and Ryther 1962) were inoculated with cell culture (*N. delicatula* or *A. coffeaeformis*) obtained from pre-existing stock cultures in the exponential growth phase. These cultures were kept at room temperature in a 12 h light: 12 h dark photocycle for 10 days. Subsequently, the cells

in the exponential phase were collected by centrifugation at 12,000 x g for 10 min and resuspended in culture medium in cryovials, at an initial concentration of 3×10^5 cells ml⁻¹.

5B.2.2 Experimental protocol

Three different protocols were employed to study the effects of low temperature (Fig.5B.1). In the first set, cryovials containing the cell culture were subjected to

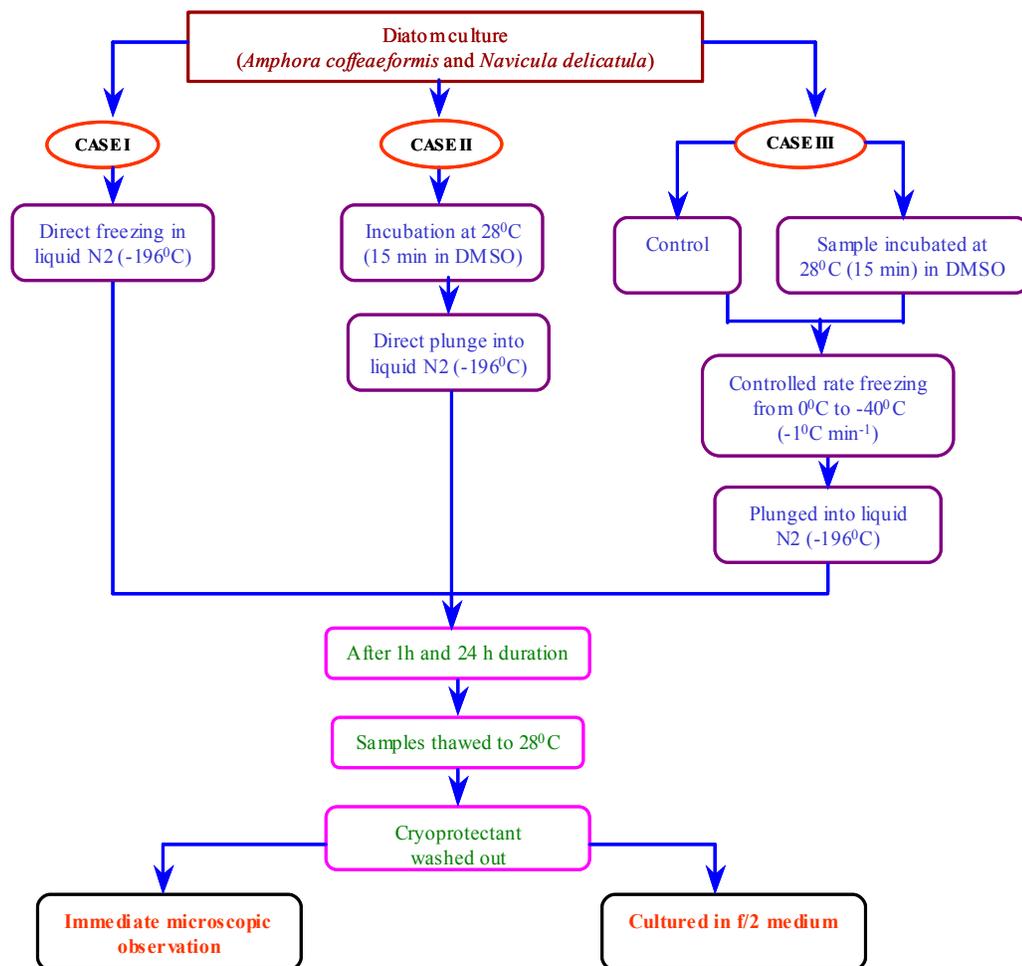


Fig. 5B.1 Schematic representation of the experimental protocol

uncontrolled direct freezing in liquid nitrogen (-196°C) and storage at the same temperature (Case I). The second set involved one-step cooling technique, in which the cryovials with cell culture were initially incubated at room temperature for 15 min

with the cryoprotectant, Dimethyl sulfoxide (DMSO). This was followed by fast cooling in liquid nitrogen (Case II). The third set involved a two-step cooling method, in which the cryovials containing a suspension of concentrated diatom cells were incubated with DMSO (2M) for 15 min at room temperature. These vials were then cooled under controlled conditions with the help of a controlled-rate freezer (Planer Model KRYO 10/16) from + 20 to 0 °C at $-5\text{ }^{\circ}\text{C} / \text{min}$ and from 0 °C to $-40\text{ }^{\circ}\text{C}$ at a rate of $-1\text{ }^{\circ}\text{C} / \text{min}$. Subsequently, these cryovials were cooled rapidly by transfer into liquid nitrogen (Case III). Control was run simultaneously without the cryoprotectant (Case III control). One set of samples was retrieved after 1 hour and another after 24 hours. Each set had four replicates, which were immediately thawed to room temperature. The samples with cryoprotectant were washed thoroughly with f/2 media to remove the cryoprotectant. Later, all the samples were cultured in fresh f/2 media.

5B.2.3 Evaluation of viability

The cells immediately after retrieval were observed for any morphological or internal changes. The same was done for the cultured cells at regular intervals to observe the cells regain their viability. Simultaneously, a control sample was kept for comparison with the treated sample during the recovery and growth of the cells. Cell numbers were also enumerated every alternate day. Staining with neutral red dye was done in order to confirm the time of regaining viability (Songdong and Jixun 2000).

5B.2.4 Data analysis

The cell abundance on incubation of *A. coffeaeformis* and *N. delicatula* after one hour and 24 hours of retrieval were subjected to multiple analyses of variance (MANOVA)

(Sokal and Rohlf 1981). Specific growth rates were calculated for both, *A. coffeaeformis* and *N. delicatula* according to Carreto and Cattogio (1976).

5B.3 Results

5B.3.1 Effects of exposure to low temperatures on *N. delicatula*

Immediate observations revealed ruptured cell chloroplasts, in case I and case II (Plate 5B.1). In case III and its control, the cell chloroplast was contracted (Plate 5B.1). On incubation, observations at regular intervals showed only the cells from case III recover wherein the contracted chloroplast showed a gradual increase in size, finally regaining the normal size and structure (Plate 5B.1). However, the growth rate of the cryopreserved cells was slower than that of the normal cells (Fig. 5B.2 and 5B.3). The cells in the case III control (i.e., without cryoprotectant) did not show any recovery (Fig. 5B.2 and Plate 5B.1).

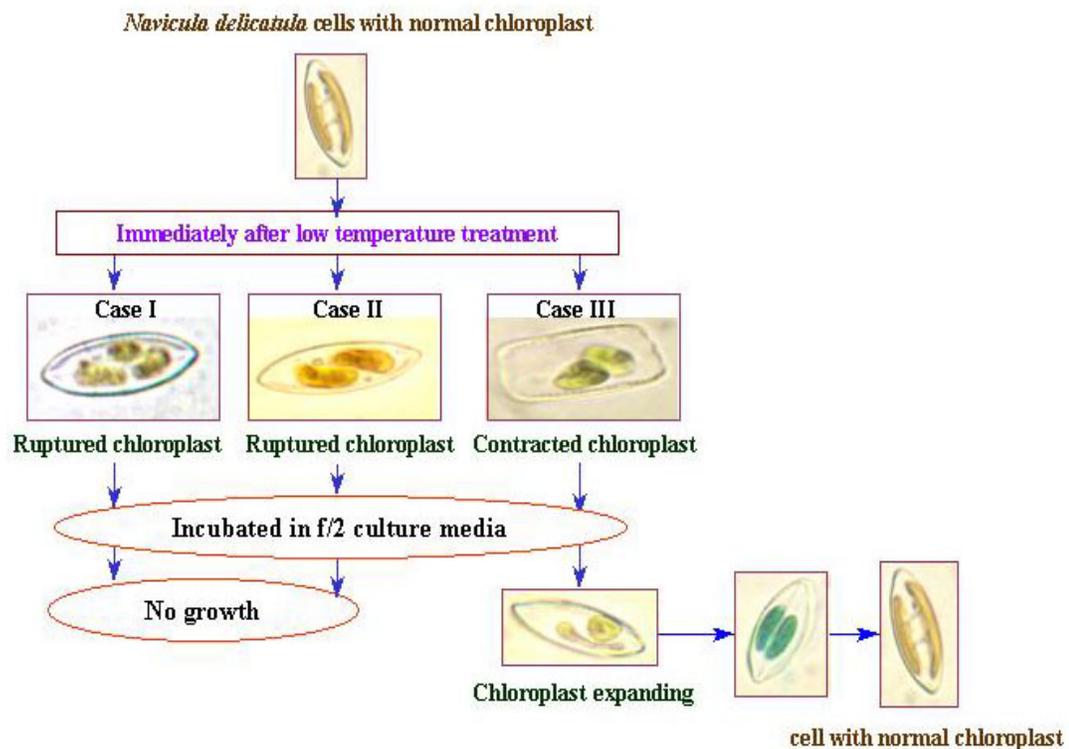


Plate 5B.1 Influence of low temperature on *Navicula delicatula*

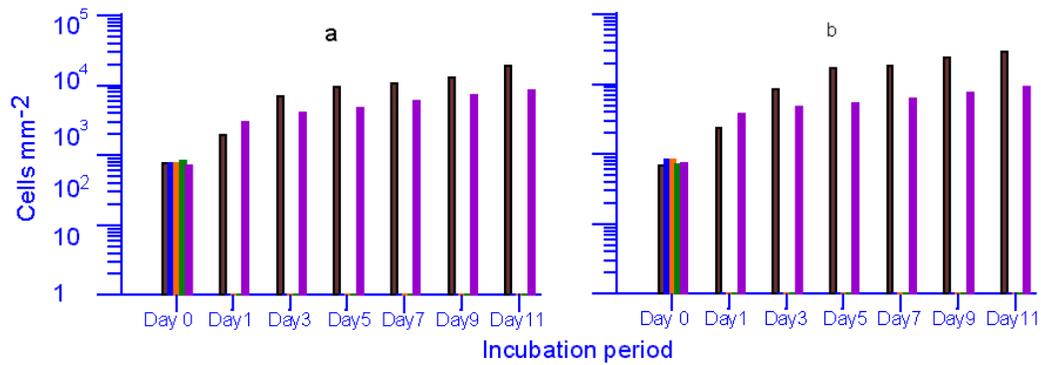


Fig. 5B.2 Cell abundance on incubation of *N. delicatula* after a period of 1h (a) and 24 h (b) of retrieval in case I, case II and case III

■ Control; ■ Case I; ■ Case II; ■ Case III control; ■ Case III

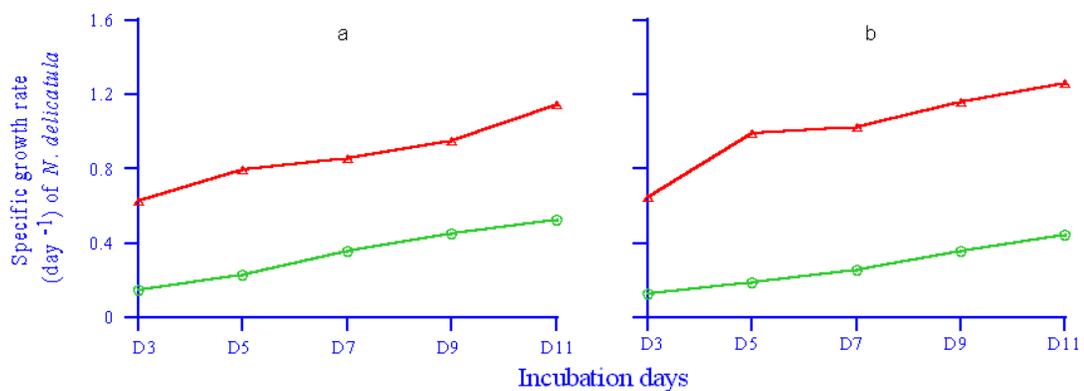


Fig. 5B.3 Specific growth rate of *N. delicatula* on incubation after a period of 1h (a) and 24 h (b) of retrieval in case I, case II and case III

△ Control; ■ Case I; ◆ Case II; ● Case III control; ○ Case III

5B.3.2 Effects of exposure to low temperatures on *A. coffeaeformis*

Immediate observations showed that the cell chloroplasts were intact in all the cases (Plate 5B.2). Similar to case III of *N. delicatula*, the chloroplasts were contracted, lying in the center of the cell. On incubation, the cells from case I, II and III regained viability, with the chloroplasts attaining the normal shape and size (Plate 5B.2 and Fig. 5B.4). This revealed the capability of cells to survive exposure to low temperatures without any cryoprotectants (Fig. 5B.4 and Fig. 5B.5).

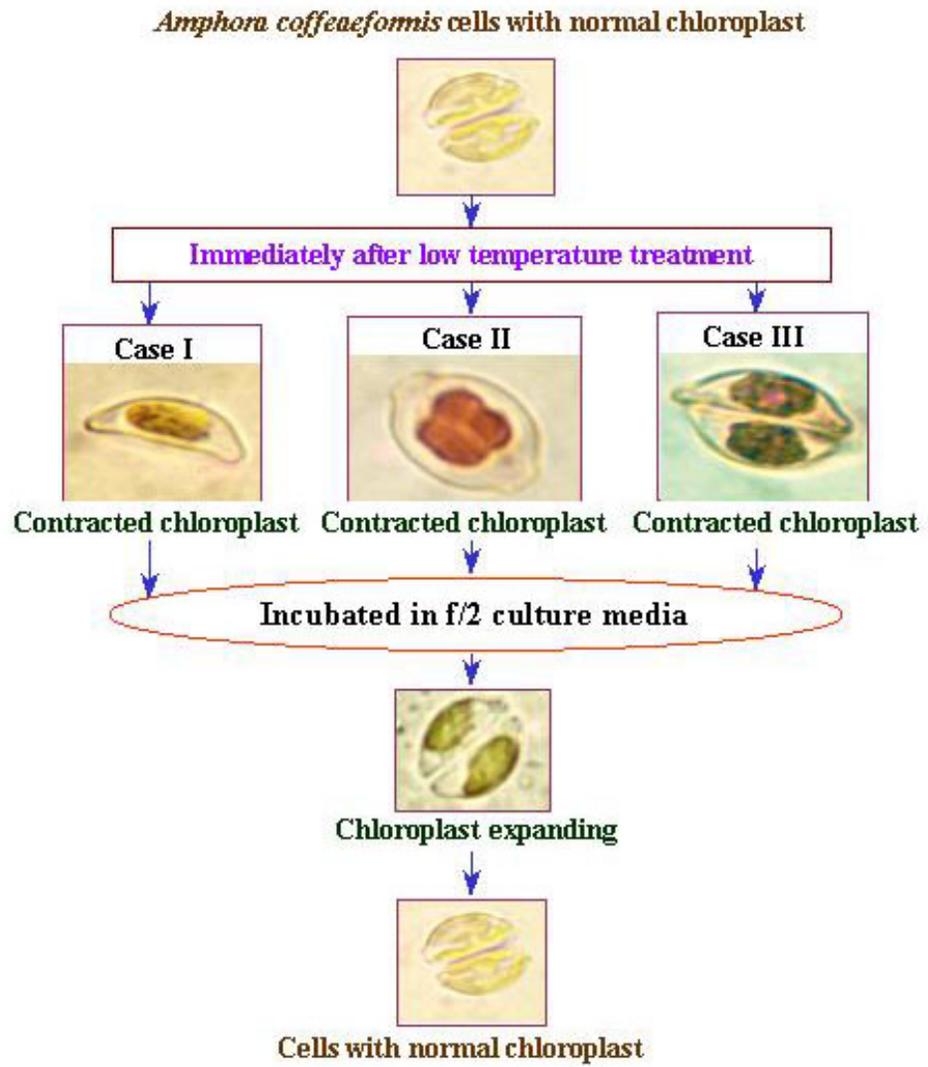


Plate 5B.2 Influence of low temperature on *Amphora coffeaeformis*

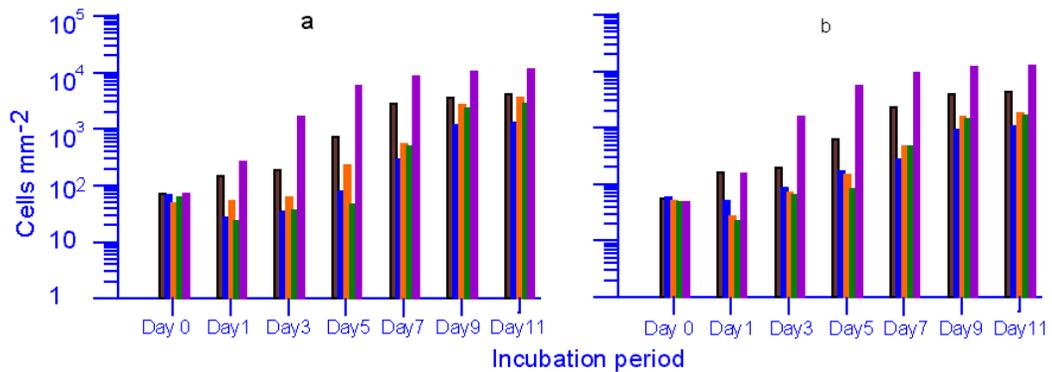


Fig. 5B.4 Cell abundance on incubation of *A. coffeaeformis* after a period of 1h (a) and 24 h (b) of retrieval in case I, case II and case III

■ Control; ■ Case I; ■ Case II; ■ Case III control; ■ Case III

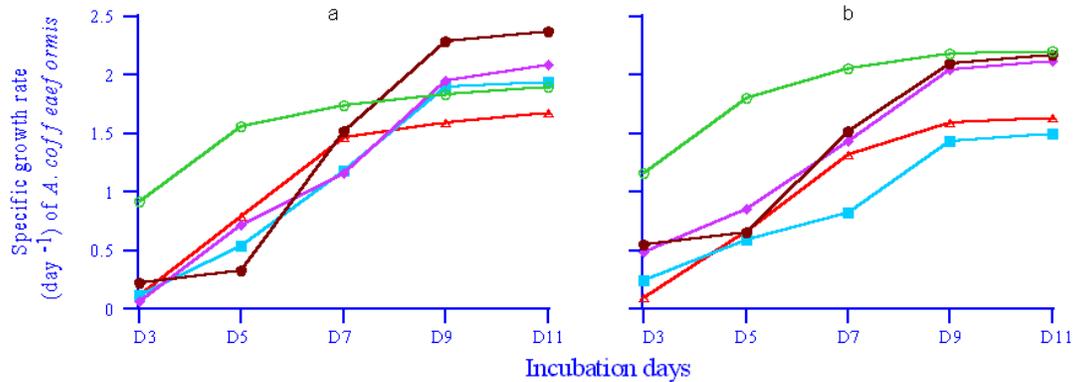


Fig. 5B.5 Specific growth rate of *A. coffeaeformis* on incubation after a period of 1h (a) and 24 h (b) of retrieval in case I, case II and case III
 Δ Control; \blacksquare Case I; \blacklozenge Case II; \bullet Case III control; \circ Case III

Table 5B.1. MANOVA (Without replication) for the variation in cell abundance between species, incubation days and experimental conditions after a duration of one hour (* $p < 0.001$; NS - not significant)

Source of variation	<i>df</i>	SS	MS	F
Diatom species	1	39.38	39.38	
Experimental condition	4	49.52	12.38	
Incubation days	5	8.42	1.68	
Diatom speciesXExperimental condition	4	4.24	1.06	20.46*
Diatom speciesXIncubation days	5	5.01	1.00	19.28*
Experimental conditionXIncubation days	20	0.57	0.03	0.54 ^{NS}
Diatom speciesXExperimental condition xIncubation days	20	1.04	0.052	

MANOVA revealed a significant variation in cell abundance between the two species with respect to incubation days and experimental conditions for the samples retrieved after one hour as well as those retrieved after 24 hours (Table 5B.1 and 5B.2).

5B.4 Discussion

A. coffeaeformis exhibited better survival capabilities as compared to *N. delicatula*, when exposed to low temperature. As Levitt (1980) has reported for higher plants, chloroplasts were the organelles showing the most striking and easily detectable change for these two species. According to Farrant et al. (1977) the reasons for the

differences among cells exposed to freezing lie first in their particular sensitivity to shrinkage and swelling under hypertonic and hypotonic conditions respectively.

Initially, when cells are subjected to subzero temperatures they supercool. The manner in which they regain equilibrium depends mainly on the rate at which they are cooled and on their permeability to water. If cooled slowly or if their permeability to water is high, they will equilibrate by dehydration. But if they are cooled rapidly or if their permeability to water is low, they will equilibrate by intracellular freezing. Thus, rapid cooling not only produces intracellular crystals it also produces small crystals, which are likely to enlarge during warming because of their high surface free energies (Mazur 1970). Thus, direct freezing into liquid nitrogen resulted in rupture of *N. delicatula* chloroplast (Case I). But this was not the case with *A. coffeaeformis*, which was viable even after direct transfer to this low temperature. This species could probably equilibrate faster by dehydration thus resulting in contracted chloroplasts.

Table 5B.2. MANOVA (Without replication) for the variation in cell abundance between species, incubation days and experimental conditions after a duration of 24 hours (* p < 0.001; NS - Not significant)

Source of variation	df	SS	MS	F
Diatom species	1	36.67	36.67	
Experimental condition	4	52.26	13.06	
Incubation days	5	7.42	1.48	
Diatom speciesXExperimental condition	4	4.25	1.06	25.92*
Diatom speciesXIncubation days	5	4.01	0.80	19.57*
Experimental conditionXIncubation days	20	0.59	0.03	0.72 ^{NS}
Diatom speciesXExperimental condition xIncubation days	20	0.82	0.04	

But regardless of whether cells equilibrate by dehydration or by intracellular freezing, freezing exposes them to loss of liquid water and to increase in concentration of intra and extracellular solutes. These events, which the cell undergoes during freezing are referred to as “solution effects” by Mazur (1970). The cells can survive if a protective additive is present. Lovelock and Bishop (1959) discovered the protective effect of

DMSO. DMSO is a protective non-electrolyte that is able to penetrate readily into cells (Smith 1950; Farrant 1965). This permeable cryoprotectant can act as a water-binding substance (Taylor 1987), reducing the amount of water molecules forming ice and, by colligative effects may reduce the osmotic stress. Most cryoprotectants appear to protect against solution effects rather than against intracellular freezing. Thus, survival of *N. delicatula* increased when cooled slowly in the presence of DMSO (Case III) as compared to that when cooled rapidly (Case II). Comparatively *A. coffeaeformis*, which is smaller in size survived even when subjected to rapid cooling (case I) and slow cooling (case III) in the absence of DMSO. Cryopreservation of marine algae without cryoprotectants has been reported in very few instances e.g., *Tetraselmis chuii*, *Nannochloropsis gadiatana*, *Nannochloropsis atomus* (Canavate and Lubian 1995a) and *Chaetoceros gracilis* (Canavate and Lubian 1994, 1995b). The survival percentage was higher in the presence of DMSO when subjected to slow cooling (Case III) which reveals the protective effect of the cryoprotectant by cell penetration and reduction of the hypertonic osmotic effects of cooling. Because, permeant DMSO replaced a proportion of the impermeant extracellular solute, less shrinkage occurred and the damage to the cells of both species was minimal.

Ben-Amotz and Gilboa (1980) has reported cryopreservation of the diatom *Phaeodactylum tricornutum* using 0.5 M DMSO while Canavate and Lubian (1994, 1995) succeeded in cryopreserving *Chaetoceros gracilis* at 1.5 M DMSO. *N. subinflata* (Redekar and Wagh 2000a) and *Chaetoceros calcitrans* (Joseph et al. 2000) were cryopreserved using 4 M and 1.5 M DMSO respectively. In the present study, 2M DMSO could successfully cryopreserve *A. coffeaeformis* and *N. delicatula* by following the two-step cooling method.

The numerical value for the critical cooling rate that produces internal ice depends on the ratio of the volume of the cell to its surface area and on its permeability to water. The critical rate should be lower for larger spherical cells and for those less permeable to water than for smaller or more permeable cells (Mazur 1970). The better survival capabilities of *A. coffeaeformis* as compared to *N. delicatula* when cooled at a rate of $-1\text{ }^{\circ}\text{C min}^{-1}$ shows that this rate of cooling was appropriate for *A. coffeaeformis*.

Significant differences between the two species in cell viability following the two-step cooling technique indicates that membrane properties / or physico-osmotic causes may contribute to cold hardening. The above results are in agreement with the general assumption that the primary site of injury to cells following freezing and thawing is the cell membrane (Mazur 1970). Differences in freezing injury among the marine algae may therefore reflect different properties and compositions of diatom membranes.

A. coffeaeformis is known to be a very sturdy diatom species capable of surviving harsh, unfavorable environmental conditions (Buzzelli et al. 1997). A few algae possess a natural capability to survive freezing better than others. The nature of freezing tolerance is not yet clear, but the ability of certain algae to survive at frost temperatures suggests that these algae have developed a cellular mechanism of freezing protection. When subjected to cold stress, Antarctic algae are known to produce a tertiary sulfonium compound, dimethyl sulfoniopropionate (DMSP). DMSP is reported to have a role to play in cryopreservation of polar algae (Karsten et al. 1990).

Species-specific genetic makeup may be responsible for such tolerance to low temperature exposures. Study of these cryoprotective proteins, which are de novo, synthesized during low temperature stress, will be of utmost importance.

5C. Immunofluorescence technique

5C.1 Introduction

Considerable time and effort are invested to identify a particular species on the basis of morphological characteristics. Often it is difficult to distinguish many features using the light microscope. An alternative to microscopic identification is the use of molecular probes, which can bind to either internal or external sites on the target species and be visualized using fluorescence techniques (Anderson 1993). Recently, immunological techniques such as species-specific (labelled) antibodies are being increasingly in use for taxonomy of bacteria and protozoa. The specificity of the antigen-antibody reaction provides a powerful tool for the study of individual microorganisms in their natural environment (Shapiro et al. 1998).

The major advantage of an immunochemical probe is that it is a highly specific and sensitive tool for investigating known systems and characterizing and localizing known antigens. One of the primary drawbacks of the immunochemical approach, however, is that, the investigator must have the culture or antigen in hand to generate the antibody probes.

Diatom taxonomy is based primarily on frustule morphology. Frustule morphology, however, can change with environmental (Schmid 1979; Mizuno 1987) and culture conditions. Immunofluorescence assays may be especially useful for diatoms for an easier and quicker identification. In the present study, an attempt was made to distinguish a pennate diatom, *Navicula delicatula*, by developing antisera directed against its cell surface antigens. *N. delicatula* is the most dominant diatom encountered in biofilms.

5C.2 Materials and methods

5C.2.1 Immunization culture

Unialgal batch cultures of the diatom *N. delicatula* were established in f/2 enriched seawater medium (Guillard and Ryther 1962) at a temperature of 27 °C under a 12 h light: 12 h dark photocycle. Cells were harvested by centrifugation at the end of exponential growth phase (7 days). The pelleted cells were preserved in 0.6% (v/v, final concentration) paraformaldehyde prepared in seawater and divided into 3 duplicate 1ml aliquots in microcentrifuge tubes each containing $\sim 5 \times 10^6$ cells of *N. delicatula* and stored at 4 °C until used for immunization (Bates et al. 1993).

5C.2.2 Test culture

Biofilms were developed by exposing stainless steel and polystyrene substrata at the Dona Paula Bay (15° 27' N 73° 48' E). These films were scraped with a nylon brush into filtered seawater. This sample was used for the immunological tests along with laboratory maintained diatom cultures (Table 5C.1). Test cultures were generally harvested during the exponential phase and preserved in 2% (v/v, final concentration) paraformaldehyde-glutaraldehyde. In addition, *N. delicatula* cells were tested either fresh, frozen or preserved as indicated in Table 5C.2, to determine the best fixation procedure for use.

5C.2.3 Immunization of rabbits

Antibodies were raised commercially (Genei India Pvt. Ltd. Bangalore, India). According to the manufacturers protocol the preserved cells were washed with phosphate-buffered saline (PBS) in order to remove the preservative. Subsequently, these cells were mixed with Freund's complete adjuvant. This mixture (0.5 ml) was

subcutaneously injected into the rabbits. The antibody titre was monitored by Dot ELISA and yielded a titre value of 1:5000.

5C.2.4 Immunofluorescence protocol

The indirect immunofluorescence (IF) protocol by Bates et al. (1993) was followed in this study (Fig. 5C.1). For the IF assay, 1ml of the preserved culture of *N. delicatula*

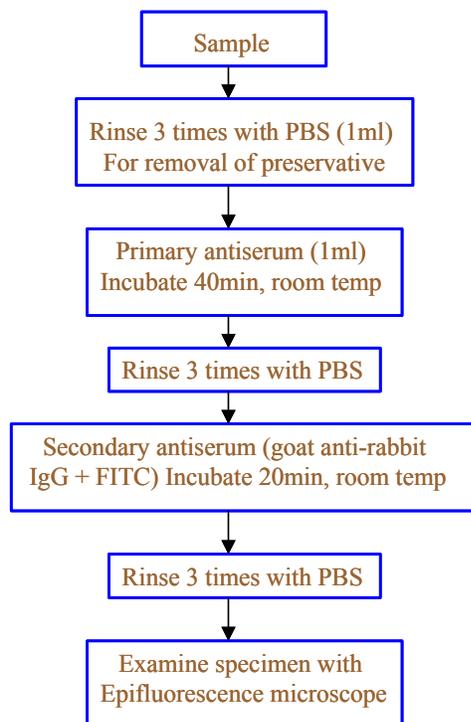


Fig. 5C.1 Experimental protocol of immunofluorescence assay

was first rinsed three times [Eppendorff microcentrifuge; 20 min at 4000 rpm) with PBS buffer (0.02 M Phosphate; 0.15 M NaCl; pH 7.45) to remove the seawater and preservative. Cells were then incubated for 40 min in the primary test antiserum, 10 times diluted with PBS. After three rinses with PBS, the cells were incubated for 20 min with 1 ml of FITC conjugated goat anti-rabbit antiserum, diluted 1:800 with PBS, as the secondary antibody and rinsed three more times with PBS to wash off the excess conjugate. One drop of this preparation was placed on a microscope slide and observed using an inverted epifluorescence microscope (Olympus IX-AN) at 100x,

using blue light excitation. To determine the composition of *N. delicatula* in the biofilm samples, first the total numbers of cells were counted through normal light microscope and then the counts of immunofluorescently labeled cells in the same field were noted.

5C.3 Results

Table 5C.1 Diatom species representing two orders of class Bacillariophyceae. Summary of results obtained by indirect immunofluorescence assay for unialgal cultures using antisera raised against *Navicula delicatula*. Cross reactivity is scaled from very strong (+++) to none (-)

S.No	Species	Family	Order	Isolated from	Cross reactivity
1	<i>Navicula subinflata</i>	Naviculaceae	Pennales	Biofilm	-
2	<i>Navicula crucicula</i>	Naviculaceae	Pennales	Biofilm	-
3	<i>Amphora coffeaeformis</i>	Amphoraceae	Pennales	Biofilm	-
4	<i>Amphora rostrata</i>	Amphoraceae	Pennales	Biofilm	-
5	<i>Skeletonema costatum</i>	Skeletonemataceae	Centrales	Water column	-
6	<i>Chaetoceros calcitrans</i>	Chaetocerotaceae	Centrales	Water column	-

In the IF assay, the antiserum successfully labelled 100% of *N. delicatula* cells from culture which was used to develop the antiserum. It also labelled those cells, which had changed their normal shape and become deformed and short due to lack of sexual reproduction. This antiserum when tested with unialgal cultures of the same genera and other species given in Table 5C.1 did not show any cross reactivity.

Table 5C.2 Comparison of various preservation techniques, including freezing on the immunofluorescent reaction between *Navicula delicatula*. Immunological cross reactivity is scaled from very strong (+++) to average (+) (Note: +++ very good, ++ Good, + Average, ab-absent)

S.No	Preservative technique	<i>Navicula delicatula</i>
1	Formalin (2%)	++
2	Glutaraldehyde (0.5%)	++
3	Paraformaldehyde (2 %)	+++
4	Paraformaldehyde/Glutaraldehyde (2 % v/v)	+++
5	Lugols Iodine (4%)	+
6	2% Paraformaldehyde (frozen at -20 ⁰ C)	+++
7	0.5 % Glutaraldehyde (frozen at -20 ⁰ C)	++
8	Control A without fixative	+
9	Control B (frozen at -20 ⁰ C) without fixative	++

Table 5C.3 Cell count of *Navicula delicatula* present in the biofilm samples developed over polystyrene and stainless steel substrata, obtained through the indirect immunofluorescence assay (PS = Polystyrene ; SS= Stainless steel)

Biofilm (n)	Total cells ml ⁻¹ normal count	cells ml ⁻¹		Stained(%)	Non Stained(%)
		Stained	Non Stained		
PS-1	2802	1528	1274	55	45
2	1528	1274	255	83	17
3	764	255	509	33	67
4	1783	255	1528	14	86
5	1019	509	509	50	50
6	1019	509	509	50	50
7	3057	1783	1274	58	42
8	3057	764	2293	25	75
9	3312	2038	1274	62	38
10	1783	764	1019	43	57
		PS Average (%)		47	53
SS-11	1528	1019	509	67	33
12	1528	1019	509	67	33
13	1783	764	1019	43	57
14	1274	764	509	60	40
15	1019	1019	0	100	0
16	1783	764	1019	43	57
17	1274	509	764	40	60
18	1019	509	509	50	50
19	2547	1783	764	70	30
20	1528	1274	255	83	17
		SS Average (%)		62	38
		Total Average (%)		54	46

Table 5C.4 Summary of the cross reactivity results obtained for field samples by indirect immunofluorescence assay using antisera raised against *N. delicatula*

Diatom species	Cross reactivity
<i>Amphora coffeaeformis</i>	-
<i>Pleurosigma angulatum</i>	-
<i>Licmophora paradoxa</i>	-
<i>Nitzschia closterium</i>	+
<i>Nitzschia longissima</i>	-
<i>Achnanthes longipes</i>	-
<i>Licmophora flabellata</i>	-
<i>Biddulphia pulchella</i>	-
<i>Nitzschia sigma</i>	-
<i>Surirella ovata</i>	-
<i>Cocconeis scutellum</i>	-
<i>Cymbella</i> sp.	+

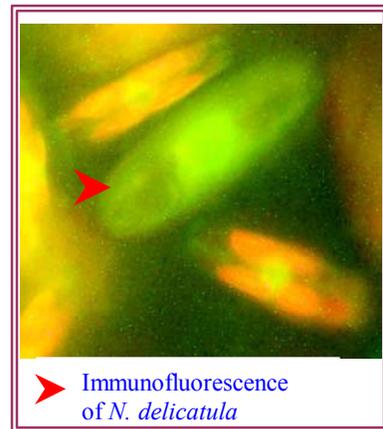
Several different preservation techniques were evaluated to determine which gave acceptable immunofluorescent results. Cells preserved with 2% (v/v, final concentration) paraformaldehyde-glutaraldehyde and 2% paraformaldehyde gave the

strongest fluorescence. *N. delicatula* cells frozen at -20°C with 2% paraformaldehyde also gave a bright fluorescence. Unfixed samples and those fixed with lugol's iodine reacted most poorly (Table 5C.2).

Among the field populations observed, on average 50% of *N. delicatula* cells were labelled by the antisera. While on stainless steel substrata, the tagging ranged from 40% to 100%, on polystyrene substrata it ranged from 14% to 83% (Table 5C.3). It showed cross reactivity with species such as *Cymbella* sp. and *Nitzschia closterium* in the biofilm samples (Table 5C.4).

5C.4 Discussion

Generally, morphological characters are used to classify diatoms to species level but increasing in the sophistication of our knowledge of non-morphological features relating to the species problem now requires a new synthesis (Taylor 1993). On the other hand the role played by genetic variability within phytoplanktonic species is unclear (Wood and Leathan



1992). In this regard, immunological procedures are promising. In this study, the primary antibody used, was produced in a rabbit in response to immunization with intact diatom cells. The antibodies are produced against the antigens located on the surface of the diatom cell.

FITC labelled antibodies have proven convenient in IF techniques with chlorophyll containing phytoplankton because both, FITC and chlorophyll are excited by blue

light, but have different emissions, FITC emitting green and chlorophyll emitting red fluorescence (Shapiro et al. 1998).

For long-term storage of sample, paraformaldehyde was the best preservative for indirect IF microscopy as found by Campbell and Itturiaga (1988) and Bates et al. (1993). Glutaraldehyde fixation not only reduces the binding capacity, resulting in a decrease of fluorescence intensity but also induces an interfering yellow-greenish background fluorescence (Campbell and Itturiaga 1988).

All of the *N. delicatula* cells reacted with the appropriate antiserum, regardless of cell size or the presence of deformities. This observation is in agreement with the results obtained by Bates et al. (1993) for *Pseudonitzschia pungens*. The same was true for *Emiliana huxleyii* (Prymnesiophyceae) (Shapiro and Campbell 1989), *Gymnodinium* and *Alexandrium* (dinoflagellates) (Mendoza et al. 1995).

On an average only up to 50% of the *N. delicatula* in field population were identified. Apparently, natural populations contain more than one serogroup. This may indicate diversity among this species wherein there is a possibility of genetic differences among clones for surface antigens. This has been demonstrated among the heterotrophic bacterial populations in the ocean (Giovannoni et al. 1990). Campbell and Carpenter (1987) observed variations in the composition of field populations of *Synechococcus* spp. by immunofluorescence, wherein ~30% of the population was labelled. Although, it is possible that physiological state of cells may alter cell surface epitopes of this diatom, Campbell et al. (1994) have shown through culture studies on eukaryotic algae that IF labelling is not affected by growth rate of the culture or by light or nutrient limitation. Campbell (1988) has reported similar results for the cyanobacterium, *Synechococcus*. Also, the physiological stage of cells did not

influence the cells of marine dinoflagellates, *Gymnodinium* and *Alexandrium* (Mendoza et al. 1995). It is also possible that the culture does not represent the strains actually present in nature (Campbell and Iturriaga 1988). Thus, it is likely that the probe is clone specific.

In this assay, slight labelling was found with other diatoms such as *Cymbella* sp. and *N. closterium*. It would be interesting to study the genetic relationship by examining the ribosomal DNA or RNA sequences of the cross reacting species. This method has already been used to obtain markers for fine-scale analysis of populations and taxonomy of *Alexandrium* (Scholin et al. 1993).

Physiologically important differences do exist among representative clones of different serogroups (Campbell and Carpenter 1987). Therefore, understanding variations of serogroups among *N. delicatula* populations is of considerable importance. Using IF assay, it is possible to define serogroups (i.e., clusters of strains labelled by one antiserum) (Campbell et al. 1983; Campbell and Carpenter 1987). Immunological tests may advance the pace of biofouling studies. They are promising in the identification, separation and enumeration of cells, but biological problems such as cross reactivity and physiological variability and technical problems i.e., signal strength, autofluorescence and loss of cells during processing must be resolved.