

## **Chapter 3**

### Diatom–bacterial interplay in benthic environments

### **3A. Can bacteria modulate diatom community structure?**

#### ***3A.1. Introduction***

Diatom-bacterial interactions are widely prevalent in the marine environment, ranging from remineralization processes (Grossart and Simon 1998) to exchange of precursors required for metabolic pathways (Puskaric and Mortain-Bertrand 2003) and domoic acid production (Bates et al. 2004). Bacteria also influence sediment stabilization by diatoms (Guerrini et al. 1998, Wigglesworth-Cooksey et al. 2001, Gerbersdorf et al. 2008).

Diatoms can regulate bacterial communities through variations in the Dissolved Organic Matter (DOM) produced during different stages of their life-cycle (Grossart et al. 2005), and through bioactive polyunsaturated aldehydes (PUAs) (Ribalet et al. 2008). Does the reverse hold true? Bacteria are known to have both, stimulatory as well as inhibitory effects on diatoms (Fukami 1997). But what about changes at the community level? Will changes in bacterial communities be reflected in diatom communities?

This study was based on the hypothesis that suppression of bacteria will result in changes in diatom community structure. This hypothesis, if supported, will have important implications in ecological terms. In the pelagic coastal system, such links between the phytoplankton and free-living and attached bacterial communities have been studied at the species composition level (Rooney-Varga et al. 2005). The present study focuses on the ability of bacteria to modulate diatom communities in the benthic system. Microphytobenthic diatom communities serve as significant

regulators of energy flow in intertidal foodwebs (Kuipers et al. 1981), and interact with bacteria to mediate sediment stabilization (Guerrini et al. 1998, Wigglesworth-Cooksey et al. 2001, Gerbersdorf et al. 2008). Most studies on microphytobenthic diatoms focus on seasonal changes, vertical migratory rhythms and other influencing factors (Admiraal and Peletier 1979, de Jonge and van Beusekom 1995, MacIntyre et al. 1996, Barranguet et al. 1998, Sundback et al. 2000, Mitbavkar and Anil 2002, 2004, 2006; Gottschalk et al. 2007, Koh et al. 2007). Very few studies (Makk et al. 2003, Haynes et al. 2007, Bruckner et al. 2008) explore the interactions between bacteria and benthic diatoms and still less is known about the community-level effects of bacteria on benthic diatoms.

There are no studies so far involving the suppression of bacteria through experimental manipulation followed by the subsequent analysis of the effects on diatom community structure, in benthic environments. This is exactly the lacuna that needs to be filled. One such method was employed in this study, wherein sediment samples were treated with different antibiotics having different modes of action – penicillin, streptomycin and chloramphenicol, individually and in combination. Penicillin inhibits cell wall peptidoglycan synthesis and is effective mainly against gram-positive bacteria (Josephson et al. 2000). Since diatoms have a different cell wall structure, penicillin cannot affect diatoms directly. Thus, any effects on diatom community structure when treated with penicillin, represents the effect of bacteria (or the indirect effect of antibiotics). Streptomycin and chloramphenicol inhibit protein synthesis, streptomycin by binding to the 30S ribosomal subunit and chloramphenicol by binding to the 50S ribosomal subunit (Josephson et al. 2000). Both have the

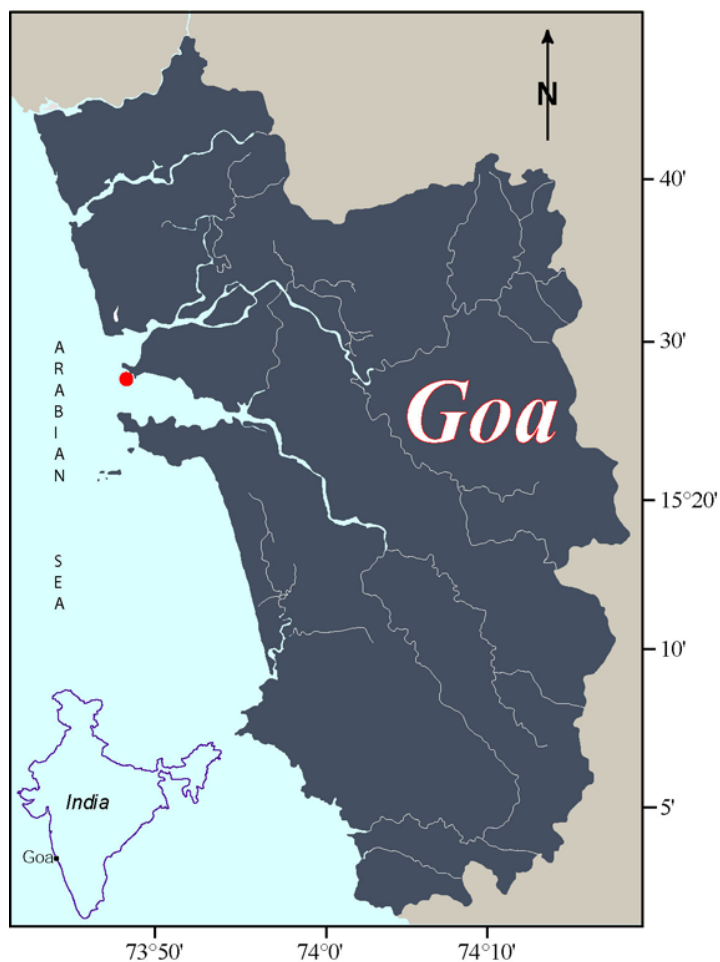
potential to affect diatoms directly by inhibiting protein synthesis in diatom organelles (70S) (Stanier et al. 1971, Cullen and Lesser 1991). In fact, chloramphenicol is a known inhibitor of photosystem II in diatoms (Ohad et al. 1984) and therefore represents the direct effect of antibiotics. Streptomycin represents the combination of potentially direct and indirect effects of antibiotics. The changes in the diatom community structure were analyzed with respect to the diatom community under control conditions.

The following aspects were investigated. (1) How does the diatom community respond to different types of antibiosis (qualitative effects), ranging from clear bacteria-mediated effects (penicillin), direct effects (chloramphenicol), to a combination of direct and indirect effects (streptomycin)? (2) How does the diatom community respond to different levels of antibiosis or bacterial suppression (quantitative effects)?

### ***3A.2. Materials and methods***

#### *3A.2.1. Study area and sampling*

Sampling was carried out in December 2006 on an intertidal sand flat at Dias Beach (15°27' N; 73° 48' E) located near Dona Paula Bay, India (Fig. 3A.1). This beach is ~ 200 m in length, sheltered on both sides by rocky cliffs and surrounded by the Mandovi and Zuari estuaries. The microphytobenthic community in this area has been extensively studied (Mitbavkar and Anil 2002, 2004, 2006).



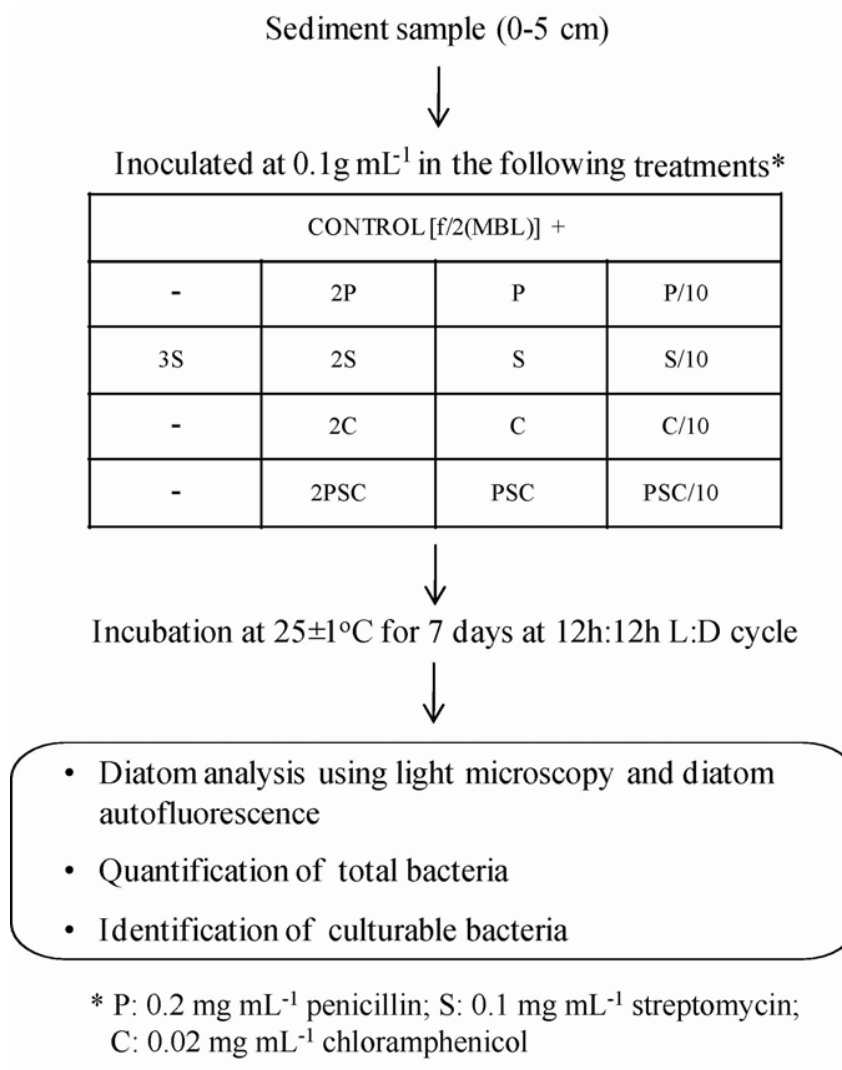
**Fig. 3A.1.** Location of the intertidal sandflat (Dias Beach) along the west coast of India.

Surface sediment (0-5 cm) was collected in triplicate from a fixed point at the mid-tide level, using PVC cores (inner diameter - 2.6 cm). Samples were stored in plastic packets (wrapped in aluminium foil to keep out light) at 5°C.

### *3A.2.2 Methodology details*

The extinction-dilution method (most-probable-number method, MPN) (Thronsen 1978) was employed for analysis of microphytobenthic diatoms. This

method involves incubation of sediment samples in appropriate media (amended with antibiotics in this study) for specific time periods, followed by identification and quantification of diatoms. Being a culture method, it facilitates the detection of algal resting stages that are often embedded in aggregates/inorganic detritus and are difficult to quantify (Harris et al. 1998).



**Fig. 3A.2.** Schematic representation of the experimental design.

Artificial seawater (MBL; developed at the Massachusetts Biological Laboratory) (Cavanaugh 1975) enriched with f/2 level of nutrients (Guillard and Ryther 1962), f/2(MBL), was used as the basal diluent, to which penicillin, streptomycin and chloramphenicol were added in varying proportions individually (P, S and C) and in combination (PSC). 3X and 2X represent high antibiotic concentrations, X and X/10 represent moderate and low antibiotic concentrations respectively; X=0.2, 0.1 and 0.02 mg mL<sup>-1</sup> for penicillin, streptomycin and chloramphenicol respectively (Fig. 3A.2).

#### *3A.2.3. Stability of antibiotics in f/2(ASW) and f/2(MBL)*

Antibiotics were dissolved at P, S, C and PSC concentrations in sterile f/2(MBL) and f/2(ASW). To analyze the possibility of change in antibiotic stability in presence of sediment, antibiotics were dissolved in sterile f/2(MBL) and f/2(ASW) containing autoclaved sediment from the study area. The flasks were incubated at 12 h: 12 h light: dark conditions at 25±1 °C for a period of 7 days. Aliquots (0.1 mL) were withdrawn on days 0 and 7 and assayed against a test culture using the well diffusion method (Elyakov et al. 1996). Wells of 1 cm diameter were bored on agar plates that were seeded with the exponentially growing test bacterial isolate. The wells were subsequently filled with 0.1 mL aliquots of antibiotic-containing f/2(MBL) and f/2(ASW). Controls were run by adding sterile distilled water, f/2(MBL) and f/2(ASW) into the wells. Assays were carried out in triplicates. The diameter of the inhibition zone (total diameter of inhibition zone minus diameter of well) was

measured after 24 h incubation at room temperature, that ranged from 26-28°C. Results are presented as zone of inhibition (percentage compared to day 0 values).

#### *3A.2.4. Analysis of microphytobenthic diatoms*

1 g sediment was suspended in the different diluents at a concentration of 0.1 g sediment mL<sup>-1</sup>. This stock was diluted ten-fold up to 10<sup>-5</sup> and 1 mL aliquots of diluted suspensions inoculated into five replicate culture wells. After incubation at 25±1 °C with a 12:12 h light : dark cycle for 7 days, diatoms were analyzed by light microscopy and confirmed as viable by observation of diatom autofluorescence. The wells in which viable diatoms were observed were scored as positive. Diatoms were identified based on Subrahmanyam (1946), Desikachary and Prema (1987), Desikachary et al. (1987a and b), Tomas (1997) and Horner (2002). Diatoms <10µm, which could not be identified to generic level, were grouped together as Unidentified Tiny Diatoms (UTDs). The MPN of diatoms in the sediment sample (MPN g<sup>-1</sup> wet sediment) was calculated by selecting a set of three consecutive dilutions and referring to a statistical table (Thronsdon 1978). The relative diatom density (cm<sup>-3</sup> wet sediment) was obtained by multiplying the MPN with the apparent specific gravity of the wet sediment (Imai and Itakura 1999), which was determined separately.

#### *3A.2.5. Quantification and identification of bacteria*

Samples were fixed with 4% formalin and counted using 4',6-diamidino-2-phenylindole (DAPI) and epifluorescence microscopy. These counts are expressed as bacteria g<sup>-1</sup> wet sediment. Culturable bacteria were isolated by spread plating



appropriate aliquots of diluted sediment on Zobell Marine Agar (ZMA) 2216 containing respective antibiotics. Morphologically distinct bacterial colonies were purified by repeated streaking on ZMA 2216 plates and maintained on ZMA 2216 slants. Bacterial isolates were identified to genus level based on morphological and biochemical tests following Bergey's Manual of Systematic Bacteriology (Holt et al. 1994).

#### *3A.2.6. Data analyses*

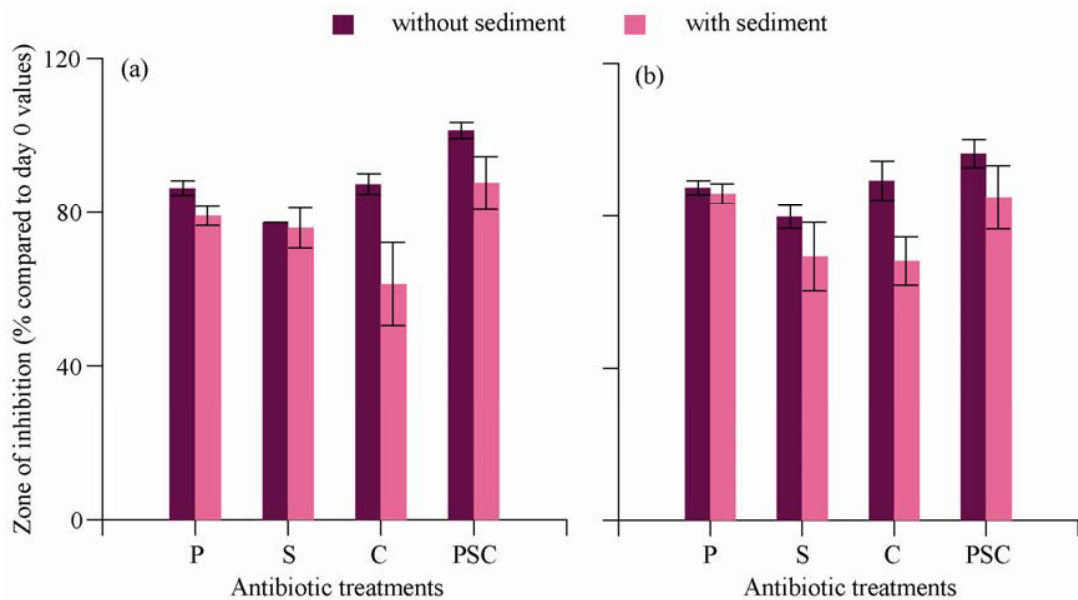
Normality and homogeneity of variances were determined using Shapiro's and Levene's tests respectively. The diatom abundance data in the control and streptomycin treatments were normally distributed and were therefore assessed for variation across the different treatments using one-way ANOVA. Diatom abundance in the other antibiotic treatments failed the requirements for parametric analysis, and were thus analyzed through the non-parametric Kruskal-Wallis test. All these analyses were performed using the STATISTICA 8 software.

Univariate measures of the diatom community that were analyzed included Shannon-Wiener's diversity index ( $H'$ ), Margalef's species richness ( $d$ ) and Pielou's evenness ( $J'$ ). Diatom abundance data were fourth root ( $\sqrt[4]{}$ )-transformed and subjected to cluster analyses. These analyses were carried out using PRIMER software (version 5).

### 3A.3. Results

#### 3A.3.1. Stability of antibiotics in *f/2*(ASW) and *f/2*(MBL)

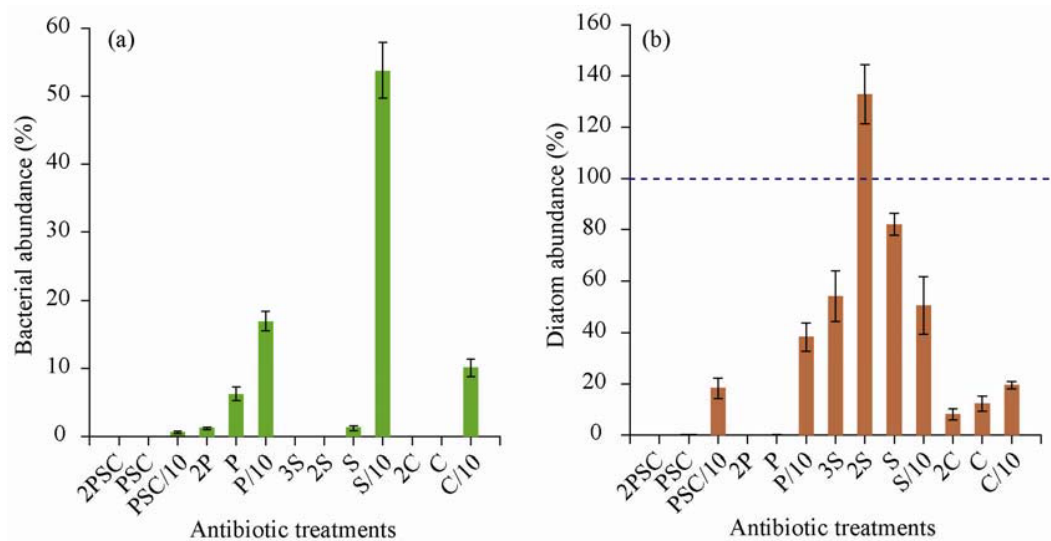
Differences in antibiotic stability/potency in the various treatments were inferred from the decrease in zone of inhibition over a 7 day period, after which diatoms are generally analyzed. The stability of antibiotics in *f/2*(MBL) ranged from 77% (S treatment) to 100% in the PSC treatment (Fig. 3A.3a). In presence of sterile sediment however, antibiotic stability was comparatively lower, ranging from 61% (C treatment) to 88% (PSC treatment) (Fig. 3A.3a). The trends observed in *f/2*(MBL) were mirrored in antibiotic treatments in *f/2*(ASW) (Fig. 3A.3b). However, the residual antibiotic activity was sufficient to suppress bacteria, even in the presence of sediment, as indicated in the results section below.



**Fig. 3A.3.** Antibiotic stability in the different treatments in the (a) CONT [*f/2*(MBL)], and (b) *f/2*(ASW), expressed in terms of zone of inhibition (percentage compared to day 0 values). Antibiotic treatment details are provided in Fig. 3A.2.

### 3A.3.2. Bacterial community

Bacterial abundance in the control diluent averaged  $2.82 \times 10^{10}$  bacteria  $\text{g}^{-1}$  wet sediment. Compared to these values, bacteria were suppressed to below detectable levels  $\leq 1\%$  in the antibiotic combination treatments (Fig. 3A.4a). Among the individual antibiotic treatments, bacteria ranged from 1-17% in the penicillin treatments,  $<1$ -12% in the chloramphenicol treatments and  $<1$ -54% in the streptomycin treatments (Fig. 3A.4a).



**Fig. 3A.4.** (a) Bacterial and (b) diatom abundance in the different antibiotic treatments, expressed as percentage of control values. Antibiotic treatment details are provided in Fig. 3A.2.

*Alcaligenes* and *Pseudomonas* (Gram-negative bacteria) and *Bacillus* (Gram-positive bacteria) were identified in the control diluent. However, no culturable bacteria were retrieved from the high and moderate antibiotic combination concentrations (2PSC and PSC); only yeast colonies were detected in these treatments. The bacteria isolated from the 2P treatment lost viability on subculturing. Shifts in the culturable bacteria were observed in the other antibiotic treatments (Table 3A.1).

**Table 3A.1.** Dominant culturable bacteria and yeast in the control and different antibiotic treatments.

	Bacteria				Yeast
	<i>Alcaligenes</i>	<i>Alteromonas</i>	<i>Bacillus</i>	<i>Pseudomonas</i>	
CONT	+		+	+	
2PSC					+
PSC					+
PSC/10	+			+	
2P		Isolates lost viability on subculturing			
P	+		+		
P/10	+	+		+	
3S	+				
2S	+			+	
S	+				
S/10	+			+	
2C	+		+		
C			+		
C/10	+	+		+	

### 3A.3.3. Diatom community

The combination of light microscopy and observation of diatom autofluorescence, used to analyze diatoms, allowed the distinction between viable and non-viable resting spores of *Coscinodiscus* and *Odontella* spp. *Cocconeis* and *Fragilariopsis* spp., which were attached to sand grains, but not visible under light microscopy, were also detected. However, *Cylindrotheca closterium*, a thinly silicified diatom, though observed to be motile under light microscopy, could not be detected through autofluorescence.

A total of 27 diatom species belonging to 14 genera were recorded in the control diluent (Table 3A.2), having an average diatom abundance of 4330 cells cm<sup>-3</sup> wet sediment (data not shown). The dominant diatoms were *Amphora rostrata* and *Amphora coffeaeformis* (Fig. 3A.5). Significant variation in diatom abundance was observed in the control diluent and the different antibiotic treatments [penicillin

treatments, Kruskal-Wallis test,  $p=0.0234$ ; streptomycin treatments, one-way ANOVA,  $p=0.0419$ ; chloramphenicol treatments, Kruskal-Wallis test,  $p=0.0237$ ; antibiotic combination treatments, Kruskal-Wallis test,  $p=0.0172$ ]. In the antibiotic combination treatments, diatom abundance varied from 100% reduction (no diatoms) in 2PSC to 99% reduction in PSC (5 diatoms  $\text{cm}^{-3}$  wet sediment) and 82% in PSC/10 treatments (532 diatoms  $\text{cm}^{-3}$  wet sediment) (Fig. 3A.4b). *A. rostrata*, *Nitzschia* sp. and UTDs dominated in PSC (Fig. 3A.5) whereas a wider range of diatoms were observed in the PSC/10 treatment (Table 3A.2). In the penicillin treatments, diatom abundance ranged from 0.1-38% (Fig. 3A.4b). The dominant diatoms differed from

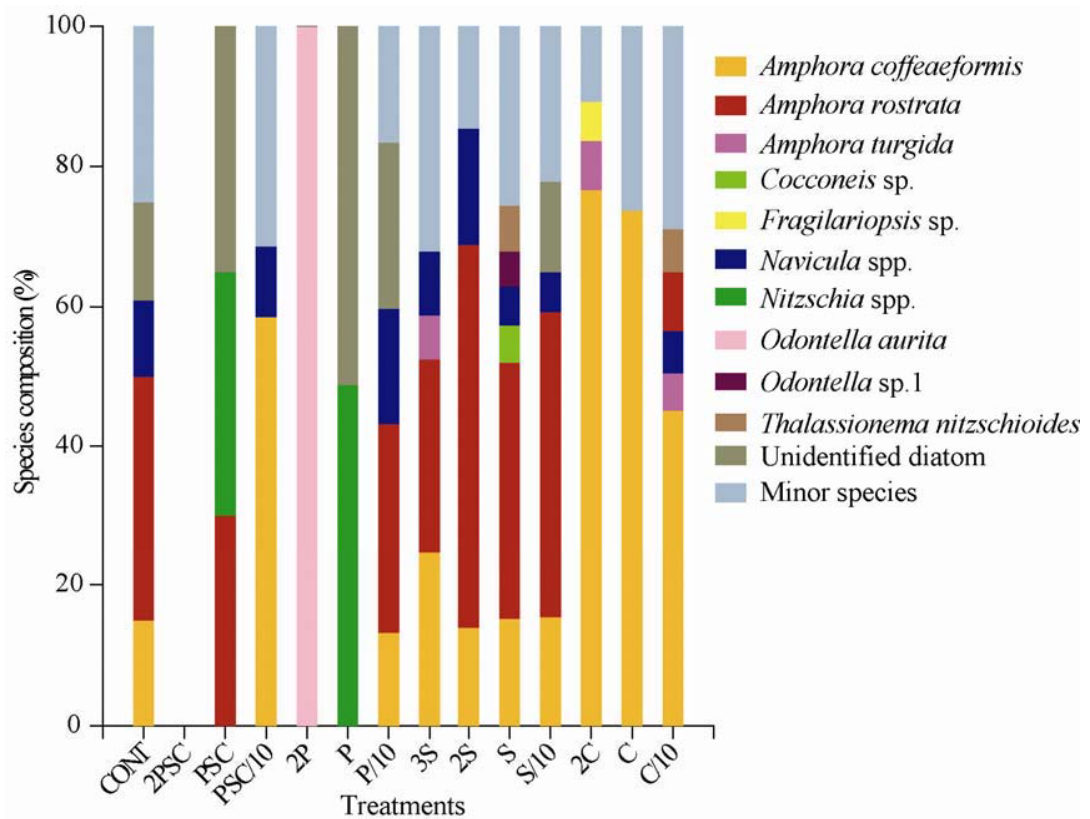
**Table 3A.2.** Diatoms observed in the different treatments.

Taxon	Treatments													
	CONT	2PSC	PSC	PSC/10	2P	P	P/10	3S	2S	S	S/10	2C	C	C/10
<b>Centrics</b>														
<i>Asteromphalus</i> sp.							+							
<i>Coscinodiscus</i> sp.	+			+			+	+	+	+	+		+	+
<i>Melosira nummuloides</i> C.A. Agardh	+							+	+		+		+	
<i>Odontella aurita</i> (Lyngbye) C.A. Agardh	+			+	+		+	+	+	+	+		+	+
<i>Odontella mobiliensis</i> (Bailey) Grunow							+	+	+	+	+			
<i>Odontella</i> sp.1	+						+	+	+	+	+	+	+	+
<i>Odontella</i> spp.	+							+	+	+	+	+	+	
<i>Thalassiosira</i> spp.	+			+			+	+	+	+	+			+

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**Table 3A.2. ...Continued**

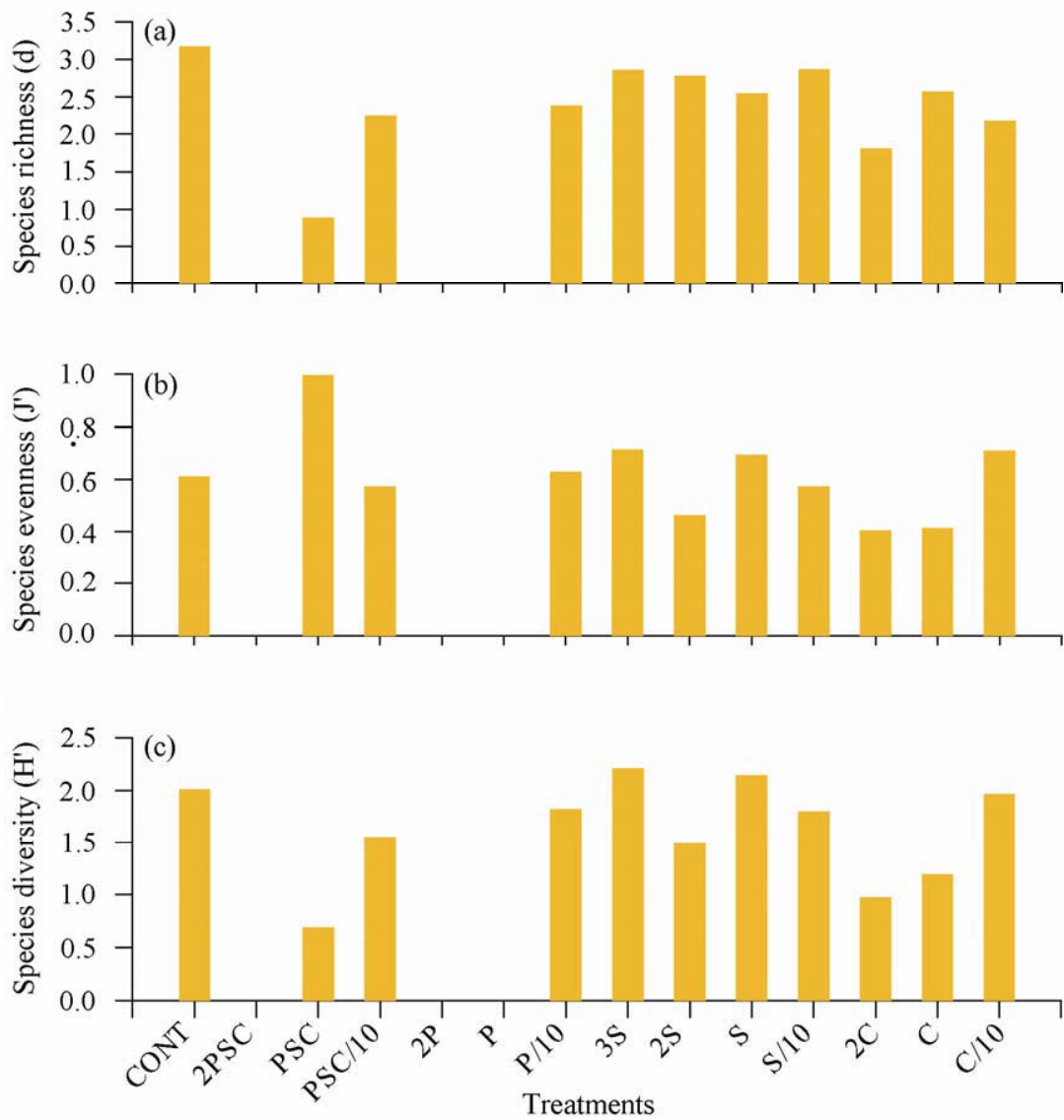
Taxon	Treatments													
	CONT	2PSC	PSC	PSC/10	2P	P	P/10	3S	2S	S	S/10	2C	C	C/10
<b>Pennates</b>														
<i>Achnanthes</i> sp.	+							+			+	+	+	
<i>Amphora coffeaeformis</i> (Agardh) Kutzing	+			+			+	+	+	+	+	+	+	+
<i>Amphora costata</i>									+	+				
<i>Amphora eunotia?</i>								+						
<i>Amphora rostrata</i> Wm. Smith	+		+	+			+	+	+	+	+			+
<i>Amphora turgida</i> Gregory	+			+			+	+	+	+	+	+	+	+
<i>Amphora</i> spp.	+			+			+	+	+	+	+		+	+
<i>Cocconeis</i> spp.	+			+			+	+	+	+	+		+	+
<i>Cylindrotheca closterium</i> (Ehrenberg) Lewin & Reimann												+		
<i>Fragilaria</i> sp.	+			+			+	+	+	+	+	+	+	+
<i>Fragilariopsis</i> spp.	+						+	+	+	+	+	+	+	+
<i>Licmophora abbreviata</i>	+													
<i>Licmophora</i> sp.											+			
<i>Navicula crucicula</i> (Wm. Smith) Donkin	+											+	+	
<i>Navicula directa</i> (W. Smith) Ralfs in Pritchard	+								+					
<i>Navicula granii</i> (Jorgensen) Gran	+								+					
<i>Navicula subinflata</i> Grunow	+			+			+	+	+	+	+			+
<i>Navicula transitans</i> var. <i>derasa</i> (Grunow, in Cleve and Grunow) Cleve	+			+			+	+	+	+	+	+	+	+
<i>Navicula transitans</i> var. <i>derasa</i> f. <i>delicatula</i> Heimdal	+								+	+	+			
<i>Navicula vanhoffennii</i> Gran	+													
<i>Navicula</i> spp.	+			+			+	+	+	+	+	+	+	+
<i>Nitzschia panduriformis</i> Gregory	+									+				
<i>Nitzschia</i> sp?	+		+	+		+	+	+	+	+	+	+	+	+
<i>Synedropsis hyperborea</i> (Grunow) Hasle, Medlin & Syvertsen	+							+	+	+	+		+	
<i>Thalassionema frauenfeldii</i> (Grunow) Hallegraeff									+					
<i>Thalassionema nitzschioides</i> (Grunow) Mereschkowsky	+			+			+	+	+	+	+		+	+
Unidentified diatoms	+		+	+		+	+	+	+	+	+	+	+	+



**Fig. 3A.5.** Species composition of the diatom communities in the control and antibiotic treatments. Antibiotic treatment details are provided in Fig. 3A.2. Minor species represent the sum of the species contributing <5%.

control and each other. *Odontella aurita* was the only diatom observed in 2P. *Nitzschia* sp. and an unidentified diatom were recorded in P whereas *A. rostrata*, *Navicula* sp. and an unidentified diatom were observed in the P/10 treatment (Fig. 3A.5). The chloramphenicol treatments supported 8-19% diatom abundance (Fig. 3A.4b). *A. coffeaeformis* was the most dominant diatom in all the chloramphenicol treatments, irrespective of the concentration, and contributed 45-77% to the diatom community (Fig. 3A.5). The streptomycin treatments did not show a linear decrease in diatom abundance with increasing streptomycin concentration. The 2S treatment

supported enhanced diatom emergence (5580 diatoms  $\text{cm}^{-3}$  wet sediment), approximately 133% compared to control (Fig. 3A.4b). *A. coffeaeformis* and *A. rostrata* dominated in all the streptomycin treatments (Fig. 3A.5).

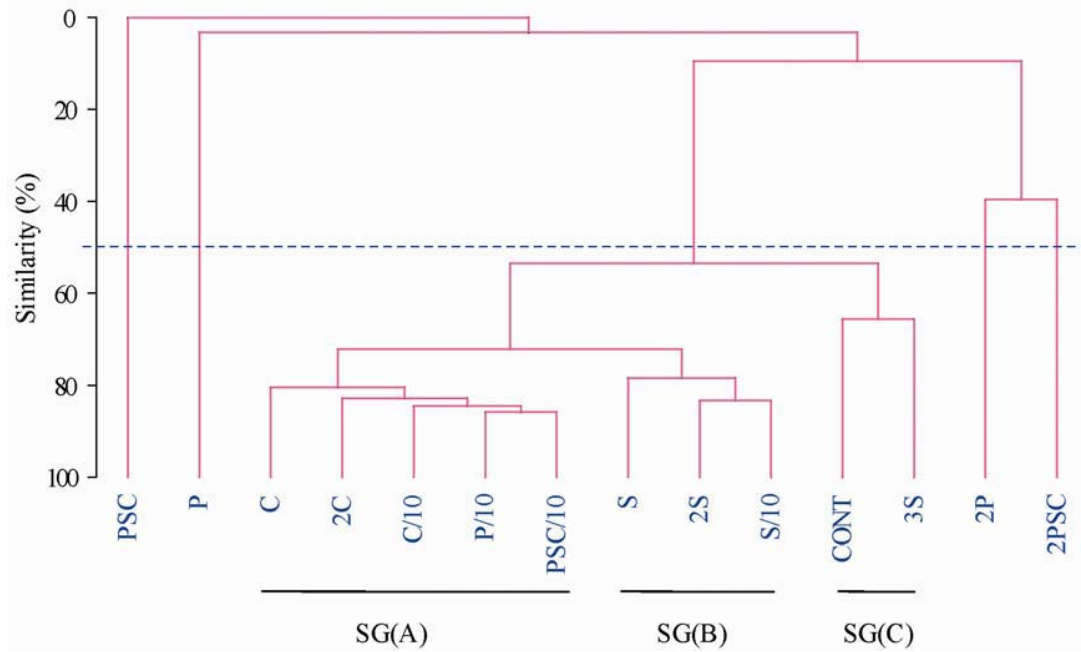


**Fig. 3A.6.** (a) Species richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the diatom community in the different treatments. Antibiotic treatment details are provided in Fig. 3A.2.

The changes in diatom community structure in the antibiotic treatments were reflected in species richness, diversity and evenness measures (Fig. 3A.6). Species



richness (d), Shannon-Wiener diversity ( $H'$ ) and Pielou's evenness ( $J'$ ) in all the antibiotic treatments was lower compared to control, except in case of diversity in 3S and S and evenness in PSC, P/10, 3S, S and S/10 (Fig. 3A.6).



**Fig. 3A.7.** Cluster dendrogram of the diatom community in the different treatments using the Bray-Curtis similarity coefficient and group average method. Antibiotic treatment details are provided in Fig. 3A.2. Dashed line (----) indicates grouping at 50% similarity. SG(A), SG(B) and SG(C) represent sub-groups A, B and C respectively.

Cluster analysis of all the treatments at 50% Bray-Curtis similarity revealed 1 group (consisting of 3 sub-groups) and 4 dissimilar treatments (Fig. 3A.7). Sub-group A consisted of CONT, 3S, 2S, S and S/10, supporting diatom abundance in the range of 1560-5580 diatoms  $\text{cm}^{-3}$  wet sediment. Sub-group B contained PSC/10, P/10 and C/10, with diatom abundance ranging from 509-1261 diatoms  $\text{cm}^{-3}$  wet sediment. Sub-group C included 2C and C treatments, with 248 and 749 diatoms  $\text{cm}^{-3}$  wet sediment respectively, and with lowest values of evenness and diversity.

The dissimilar treatments (2PSC, 2P, P and PSC) supported no diatoms/very low diatom abundance.

#### **3A.4. Discussion**

In absence of sediment, antibiotic stability patterns were identical in f/2(MBL) and f/2(ASW) (Fig. 3A.3). The antibiotic combination was the most stable. Among individual antibiotic treatments, chloramphenicol was the most stable, followed by penicillin and streptomycin. However, chloramphenicol was the least stable in the presence of autoclaved sediment. In fact, antibiotic stability was comparatively lower in all the treatments in presence of autoclaved sediment. Stability of antibiotics in a seawater-based medium depends on the combined effect of many factors including photodegradation and interaction with ions in seawater (abiotic process), adsorption to sediment (physical process) and susceptibility to microbial attack (biotic process). For e.g., after photochemical degradation, oxytetracycline and quinolones form divalent cationic complexes with  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in seawater, which results in a loss of antibacterial activity in less than one month through cation complexation or binding to sediment or associated organic matter, or a combination (Hernandez Serrano 2005).

Penicillin, a  $\beta$ -lactam antibiotic, is inactivated due to a combination of microbially-induced chemical and enzymatic hydrolyses (Gavalchin and Katz 1994). Streptomycin gets completely adsorbed onto the clay fraction of soil (Gavalchin and Katz 1994). In this study, presence of autoclaved sediment lowered streptomycin stability by 1% in f/2(MBL) and by 11% in f/2(ASW) compared to antibiotic stability values in absence of sediment (Fig. 3A.3). Since autoclaved sediment was used in

these experiments, microbial degradation of antibiotics can be ruled out. This indicates that the lowered antibiotic stability in presence of autoclaved sediment is mainly due to adsorption of antibiotics onto the 'very fine sand' (63-124  $\mu\text{m}$ ) grain size fraction of the sediment that is abundant in the study area (Mitbavkar and Anil 2002).

A wide range of bacterial suppression, from below detectable levels to 54% was obtained through the usage of different concentrations of antibiotics, individually and in combination. Significant changes in the abundance, species composition, species richness, diversity and evenness of the diatom communities in the different antibiotic treatments were observed simultaneously (Figs. 3A.4-3A.6). No diatoms were observed in the 2PSC treatment, where bacteria were eliminated beyond detectable levels (Fig. 3A.4). Diatom abundance was reduced in the moderate (upto 99%) and low antibiotic concentrations (upto 82%) (Fig. 3A.4b) with characteristic shifts in dominant diatoms (Fig. 3A.5). This was in sharp contrast to the control diluent that supported a wide range of diatoms. Among the antibiotic combination treatments, culturable bacteria were detected only in PSC/10 and consisted mainly of *Alcaligenes* and *Pseudomonas* spp. The isolates from the high and moderate antibiotic combination were identified as yeasts. Yeasts are important members of the microbial community in soil and have diverse degradative abilities (Slavikova and Vadkertiova 2003). The elimination of bacteria to below detectable levels or very low levels when treated with 2PSC and PSC favoured the development of these organisms. Examples of suppression of bacteria leading to emergence of other groups abound. For example, thraustochytrids were observed in the high concentration penicillin treatment (2P), that showed reduced diatom emergence.

Also, when diatom cultures are treated with antibiotics to suppress bacterial growth, they become susceptible to fungal pathogenic attack (personal observation). In both these cases, diatom growth is severely inhibited. This correlates well with the observations in the PSC and 2PSC treatments, i.e., elimination of bacteria and dominance of yeasts were associated with 99-100% reduction in diatoms (Fig. 3A.4). This suggests the possible involvement of bacteria in modifying the interactions of diatoms vis-a-vis other organisms.

Among the individual antibiotic treatments, marked shifts in species composition of the diatom community were observed in the penicillin treatments (Fig. 3A.5), which reduced diatoms to 62-99.9% (Fig. 3A.4b). Only one diatom species (*O. aurita*) was observed in the high penicillin concentration (Fig. 3A.5; Table 3A.2). This tychopelagic, centric diatom, usually dominates when competition in the form of other diatoms is absent. This is observed in higher dilutions or when growth of other diatoms is suppressed (personal observation). Penicillin G inhibits mainly Gram-positive bacteria (Josephson et al. 2000), which are surprisingly diverse in coastal ecosystems (Holt et al. 1994). Gram-positive bacteria were originally thought to comprise a minor fraction (5%) of bacterial communities in the ocean (Zobell 1946). However, recent research involving the use of low-nutrient media (Stevenson et al. 2004, Gontang et al. 2007, Stevens et al. 2007), has revealed that the abundance and diversity of Gram-positive bacteria in sediments may be considerably greater. They play prominent roles in coastal ecosystems. They have the ability to degrade various biopolymers (Gontang et al. 2007, Stevens et al. 2007) and are prolific producers of

bioactive metabolites (Berdy 2005). In view of the results of this study, it is possible that this group of bacteria play a significant role.

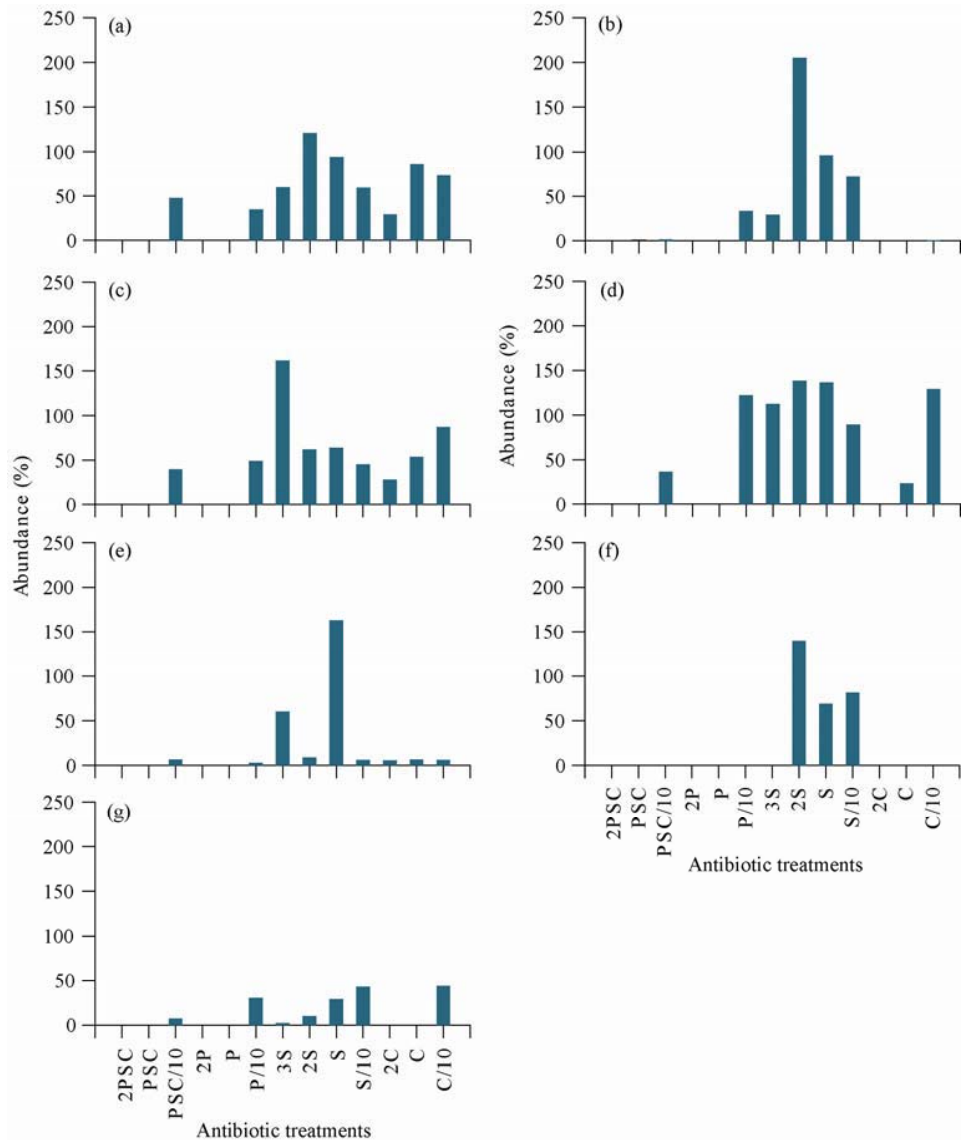
Streptomycin and chloramphenicol are relatively broad-spectrum (Josephson et al. 2000), affecting both Gram-positive and Gram-negative bacteria (which are major components of culturable marine bacteria (Jensen and Fenical 1995) and also have the potential to affect diatoms directly. Yet, diatom community structure differed in both these sets of treatments. The streptomycin treatments showed variations in diatom emergence, with the 2S treatment supporting maximum diatom abundance, higher than even the control diluent (Fig. 3A.4b). All the streptomycin treatments supported diatom communities similar to that in the control diluent; this was evident in cluster analysis (Fig. 3A.7) and one-way ANOVA ( $p=0.0419$ ). In the chloramphenicol treatments, diatom abundance was reduced to 8-19% (Fig. 3A.4b), with *A. coffeaeformis* being the dominant diatom, irrespective of the concentration (Fig. 3A.5).

From the above, it is clear that among the individual antibiotics, penicillin is the sole antibiotic that affects diatoms only through bacteria. This is because penicillin affects peptidoglycan synthesis in bacteria (Josephsen et al. 2000), and diatoms have a different cell wall structure (Tomas 1997). Yet, marked differences in diatom abundance and species composition were observed in the penicillin treatments (Figs. 3A.4, 3A.5), indicating that bacteria do affect diatom community structure. Compared to the other antibiotics, penicillin supported higher bacterial abundance, in the range of 1-6% in the high and moderate concentrations (Fig. 3A.4a). This could be due to either the concentration of penicillin used or its mode of action. Penicillin

has been used at the highest range of antibiotic concentrations among all the three antibiotics (Fig. 3A.2). Thus, the plausible reason for the higher bacterial abundance in the penicillin treatments, compared to the other treatments, could be its mode of action, i.e., the selective inhibition of mainly Gram-positive bacteria by penicillin. However, a Gram-positive bacterium, *Bacillus* sp., was recorded in the moderate concentration penicillin treatment, suggesting the importance of the concentration of penicillin used.

All the individual low concentration treatments (P/10, S/10 and C/10) supported 10-54% bacterial abundance. On cluster analysis with respect to the diatom community, P/10, C/10 and PSC/10 clustered together whereas S/10 clustered along with the control and other streptomycin treatments (Fig. 3A.7). It is tempting to speculate that the lower levels of bacterial mortality in the low concentration treatments may be linked to the higher emergence levels of diatoms. However, the changes in the diatom community when treated with antibiotics did not mirror the trends observed in bacterial abundance. This is especially noticed in case of streptomycin; diatoms did not show a dose-dependent response to different levels of streptomycin, in other words, to different levels of bacterial suppression. This disparity in trends for diatoms and bacteria suggests that differences in the diatom communities in specific antibiotic treatments cannot be directly related to reduction in bacterial abundance alone. Species-specific changes in the bacterial community, dependent on the mode of action and the concentration of antibiotics used, may possibly play an important role. An indication of this is illustrated in the shift in culturable bacteria in the antibiotic treatments (Table 3A.1). However, culturable

bacteria comprise a minute fraction of the total bacterial population and any trend in occurrence and distribution of culturable bacteria cannot be extrapolated to total bacterial populations. Therefore, accurate information about species-specific changes in the total bacterial community cannot be stated clearly at this stage.



**Fig. 3A.8.** Distribution of (a) *Amphora coffeaeformis*, (b) *A. rostrata*, (c) *A. turgida*, (d) *Coscinodiscus* sp., (e) *Navicula transitans* var. *derasa*, (f) *N. transitans* var. *derasa* f. *delicatula* and (g) *Thalassiosira* sp. in the different antibiotic treatments, expressed as percentage compared to control. Antibiotic treatment details are provided in Fig. 3A.2.

*Amphora* and *Navicula* spp. were sensitive to high and moderate concentrations of penicillin, indicating the relevance of penicillin-sensitive bacteria (mainly Gram-positive species) to these diatoms. However, they were abundantly recorded in the streptomycin treatments, in some instances, at levels far higher than that in control (Fig. 3A.8). This was in spite of suppression of bacteria to <1% of control values (Fig. 3A.4a), a shift in culturable bacteria (Table 3A.1) and the potential of streptomycin to inhibit diatoms directly. Possibly, the particular subset of bacteria inhibited by streptomycin, is not necessary for diatom growth. Or else, bacterial inhibition by streptomycin relaxes the constraints on diatom growth, resulting in higher diatom abundance than that in the control.

Summarizing the results, characteristic shifts in diatom community structure were recorded in the penicillin treatments, which affected diatoms indirectly, by inhibiting a subset of bacteria containing mainly Gram-positive species. These effects are distinct from those of the other antibiotic treatments (streptomycin, chloramphenicol and the antibiotic combination). These observations highlight the possible involvement of bacteria as a regulatory factor in diatom dynamics.



### **3B. What are the concurrent changes in the bacterial community?**

#### ***3B.1. Introduction***

Diatoms share a close but ambiguous relationship with bacteria. In addition to being symbiotic, diatom-bacterial interactions may also be antagonistic (Cole 1982, Fukami et al. 1997, Croft et al. 2005, Grossart and Simon 2007). Bacteria also influence various processes mediated by diatoms such as production of exudates, capsule formation, etc. which modulate the growth and metabolic activities of diatoms (Bruckner et al. 2008). Bacteria influence the strength by which diatoms adhere to a substratum (Grossart 1999, Wigglesworth-Cooksey and Cooksey 2005). They also influence sediment stabilization by diatoms (Wigglesworth-Cooksey et al. 2001, Gerbersdorf et al. 2008a and b).

Sediment samples were incubated with antibiotics with different modes of action – penicillin (a  $\beta$ -lactam antibiotic which cannot affect diatoms directly), streptomycin (an aminoglycoside) and chloramphenicol (a known inhibitor of diatom photosynthesis) (Ohad et al. 1984). Changes in the diatom community were analyzed through microscopy; the bacterial component was analyzed through culture techniques and Denaturing Gradient Gel Electrophoresis (DGGE) profiling.

The ARB (antibiotic-resistant bacteria) fraction can tolerate and multiply in the presence of the particular antibiotic, whereas the ATB (antibiotic-tolerant bacteria) fraction represents those bacteria that can tolerate but not divide in the presence of the antibiotic. The ATB fraction represent a seed population that can give rise to a new population with normal susceptibility once the antibiotic is removed (Keren et al. 2004).

*Vibrio* spp. are closely associated with phytoplankton (Seeligmann et al. 2008). *Vibrio proteolytica* feeds on exudates from *Amphora coffeaeformis* (Murray et al. 1986). Another species, pathogenic *Vibrio cholerae*, responsible for cholera epidemics worldwide (Colwell 1996), survives unfavourable inter-epidemic periods through existence in its Viable Non-Culturable (VNC) form in biological reservoirs such as phytoplankton (Eiler et al. 2006). In the natural environment, the proliferation of *V. cholerae* is controlled by food web effects (Worden et al. 2006) and antagonistic interactions among marine bacteria (Long et al. 2005). *Vibrio parahaemolyticus* is less deadly, but causes food-borne illnesses and can result in pandemic outbreaks of disease (Wong et al. 2000). It shows enhanced survival when adsorbed onto plankton (Sarkar et al. 1985).

In view of the above, the following aspects were investigated. (1) What are the concurrent changes in diatom and bacterial communities when treated with penicillin and other antibiotics? (2) What are the changes in antibiotic-resistant and antibiotic-tolerant bacteria? (3) What are the corresponding changes in *Vibrio* dynamics?

### **3B.2. Materials and methods**

#### *3B.2.1. Study area and sampling*

Sampling was carried out in May 2008 on a sand flat at Dias Beach (15°27' N; 73° 48' E) (Fig. 3A.1 on page 60) located near Dona Paula Bay and surrounded by the Mandovi and Zuari estuaries. The microphytobenthic diatom community in this area has been studied in detail (Mitbavkar and Anil 2002, 2004, 2006). Surface sediment (0-5 cm) was collected in triplicate at the mid-tide level using PVC cores (inner diameter 2.6 cm). Separate sediment cores were collected for analysis of specific

gravity. Samples were stored in plastic packets (wrapped in aluminium foil to keep out light) at 5°C.

### *3B.2.2. Analysis of diatoms*

The extinction-dilution method (most-probable-number method, MPN) (Thronsdon 1978), used for analyzing diatoms was modified by addition of antibiotics, individually and in combination. Nutrient enrichment (f/2) medium (Guillard and Ryther 1962) prepared in artificial seawater (MBL) (Cavanaugh 1975) was used as the control diluent (CONT). f/2 medium prepared in aged seawater [f/2(ASW)] was used as an additional control for comparison. Penicillin, streptomycin and chloramphenicol were added to the control diluent in the following concentrations: 0.2 mg mL<sup>-1</sup> (penicillin-P), 0.1 mg mL<sup>-1</sup> (streptomycin-S) and 0.02 mg mL<sup>-1</sup> (chloramphenicol-C) individually and in combination (PSC). Additional studies revealed that streptomycin seemed to enhance diatom growth. Therefore, additional streptomycin treatments [0.3 mg mL<sup>-1</sup> (3S), 0.2 mg mL<sup>-1</sup> (2S), and 0.01 mg mL<sup>-1</sup> (S/10)] were used. These antibiotic concentrations are in the range used to make diatom cultures axenic (Patil and Anil 2005).

Sediment (1 g) was suspended in respective diluents at a concentration of 0.1 g sediment mL<sup>-1</sup>, diluted up to 10<sup>-5</sup> and 1 mL aliquots inoculated into five replicate culture wells. After incubation at 25±1 °C with a 12:12 h light : dark cycle for 7 days, diatoms were analyzed by light microscopy and confirmed as viable by observation of diatom autofluorescence. The wells in which viable diatoms were observed were scored as positive. Diatoms were identified based on Desikachary and Prema (1987), Desikachary et al. (1987a and b), Horner (2002), Subrahmanyam

(1946) and Tomas (1997). The Most Probable Number (MPN) of diatoms in the sediment sample (MPN g<sup>-1</sup> wet sediment) was calculated by selecting a set of three consecutive dilutions and referring to a statistical table (Thronsen 1978). The relative diatom density (cm<sup>-3</sup> wet sediment) was obtained by multiplying the MPN with the apparent specific gravity of the wet sediment (Imai and Itakura 1999), which was determined separately.

### *3B.2.3. DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) profiling*

The changes in bacterial community structure in the different treatments were determined through Denaturing Gradient Gel Electrophoresis (DGGE) profiling. The diluents were carefully decanted from the sediment samples which were then frozen till extraction of nucleic acids followed by PCR-DGGE. DNA from the sediment samples was extracted using Powermax<sup>TM</sup> soil DNA isolation kit (MO BIO, USA) and subjected to PCR. The primers used for PCR amplification of 16S rRNA gene fragments were 341F-GC (containing a 40-bp GC-rich sequence at the 5P-end) and 907RM, which is an equimolar mixture of the primers 907RC (5P-CCGTCAATTCCTTTGAGTTT-3P) and 907RA (5P-CCGTCAATTCATTTGAGTTT-3P) (Integrated DNA Technologies, USA). The PCR conditions were as follows – denaturation of template DNA at 94 °C for 5 mins followed by 95 °C for 1 min; annealing of the template DNA at 55 °C for 1 min; extension of primers at 72 °C for 1 min; and a final extension at 72 °C for 5 mins. PCR products were inspected on 2% (w/v) agarose gels to check for amplification and subsequently subjected to DGGE analyses with a DCode system (Bio-Rad) using

a denaturing gradient of 20-80% denaturants (urea and formamide) (Schafer and Muyzer 2001). The DGGE analysis was carried out at Disha Institute of Bio-Technology Private Limited, Nagpur.

The DGGE gel was analyzed using Quantity One (version 4.66, Bio-Rad, USA). Bands were detected using band finding algorithm available with the software. The gel was also manually examined to match the bands that were not identified by the software. The band pattern was converted into a presence-absence matrix and subjected to cluster analysis based on the Bray-Curtis similarity coefficient using PRIMER (version 5).

#### *3B.2.4. Quantification of antibiotic-resistant bacteria (ARB) and antibiotic-tolerant bacteria (ATB)*

0.1 mL aliquots (in triplicate) were withdrawn daily from the control and antibiotic treatments throughout the 7 day incubation period and spread plated on appropriate media. Zobell Marine Agar (ZMA 2216) with antibiotics was used for enumerating antibiotic-resistant bacteria (ARB). ZMA 2216 without antibiotics was also used; the difference in bacterial counts on both media represented the antibiotic-tolerant bacteria (ATB).

### 3B.2.5. Quantification of *Vibrio* spp.

Thiosulphate citrate bile salts sucrose (TCBS) agar was used as the plating medium. This medium allows the quantification of total vibrios, *V. cholerae* (sucrose-fermenting, yellow, <2 mm dia colonies) and *V. parahaemolyticus* (bluish-green, ~ 1 mm dia colonies). When appropriate aliquots were plated on TCBS medium, *V. parahaemolyticus* were observed to dominate. Few yellow colonies were observed when lower dilutions were plated out. However, these could not be quantified due to matt growth of *V. parahaemolyticus*. Hence, only *V. parahaemolyticus* were quantified and are reported here.

### 3B.2.6. Data analyses

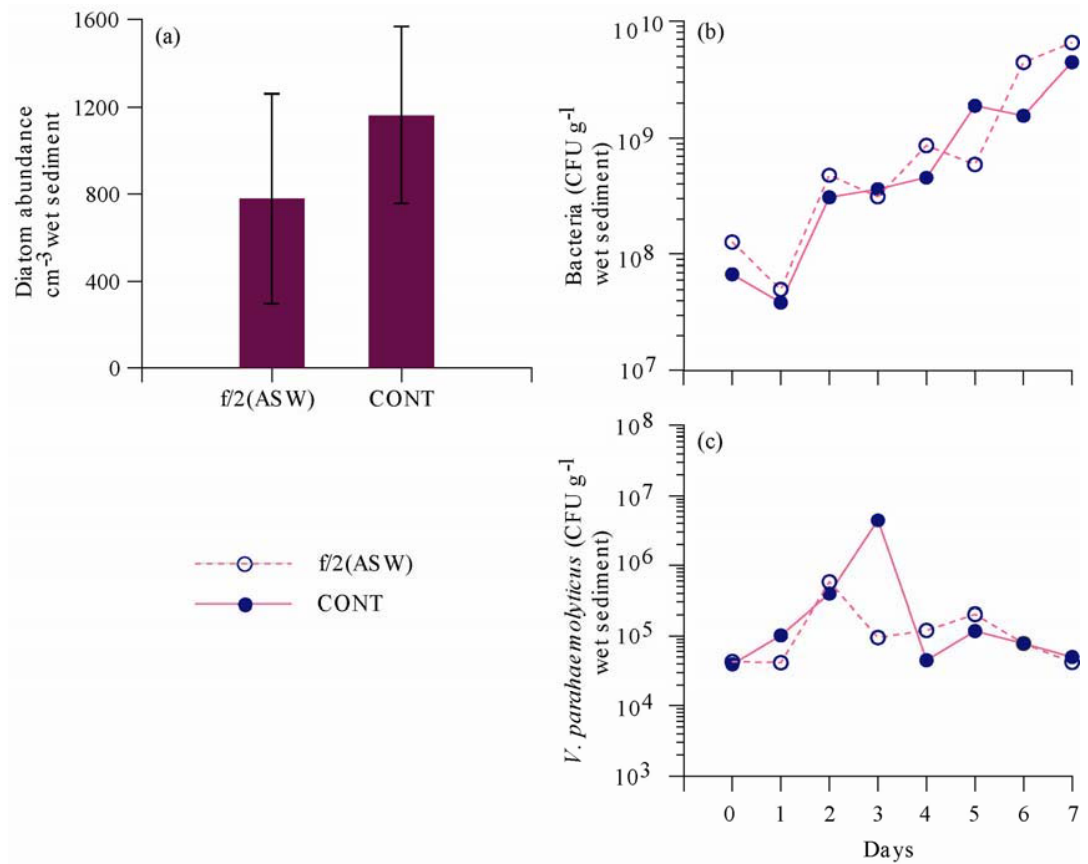
Normality and homogeneity of variances were determined using Shapiro's and Levene's tests respectively. Diatom and ARB, ATB and VP abundance data failed the requirements for parametric analysis, and were thus analyzed through the non-parametric Kruskal-Wallis test. For analysis of the diatom abundance data, the antibiotic treatments were considered separately - set I (CONT, P, S, C and PSC) representing qualitative effects and set II (CONT, 3S, 2S, S and S/10) representing quantitative effects. For analysis of the bacterial abundance data, the significance of variation between the control conditions and individual antibiotic treatments over the entire incubation period was evaluated. All these analyses were performed using the STATISTICA 8 software.

Univariate measures of the diatom community i.e., Shannon-Wiener's diversity index ( $H'$ ), species richness ( $d$ ) and evenness ( $J'$ ), were calculated. Diatom abundance data was also fourth root ( $\sqrt[4]{\cdot}$ )-transformed and subjected to cluster analyses. The DGGE band pattern was transformed into a presence/absence matrix using Quantity One software by BioRAD and subjected to cluster analysis. Cluster analysis was carried out through Bray-Curtis coefficients and the group average method using PRIMER software version 5.

### **3B.3. Results**

#### *3B.3.1. Diatom and bacterial communities*

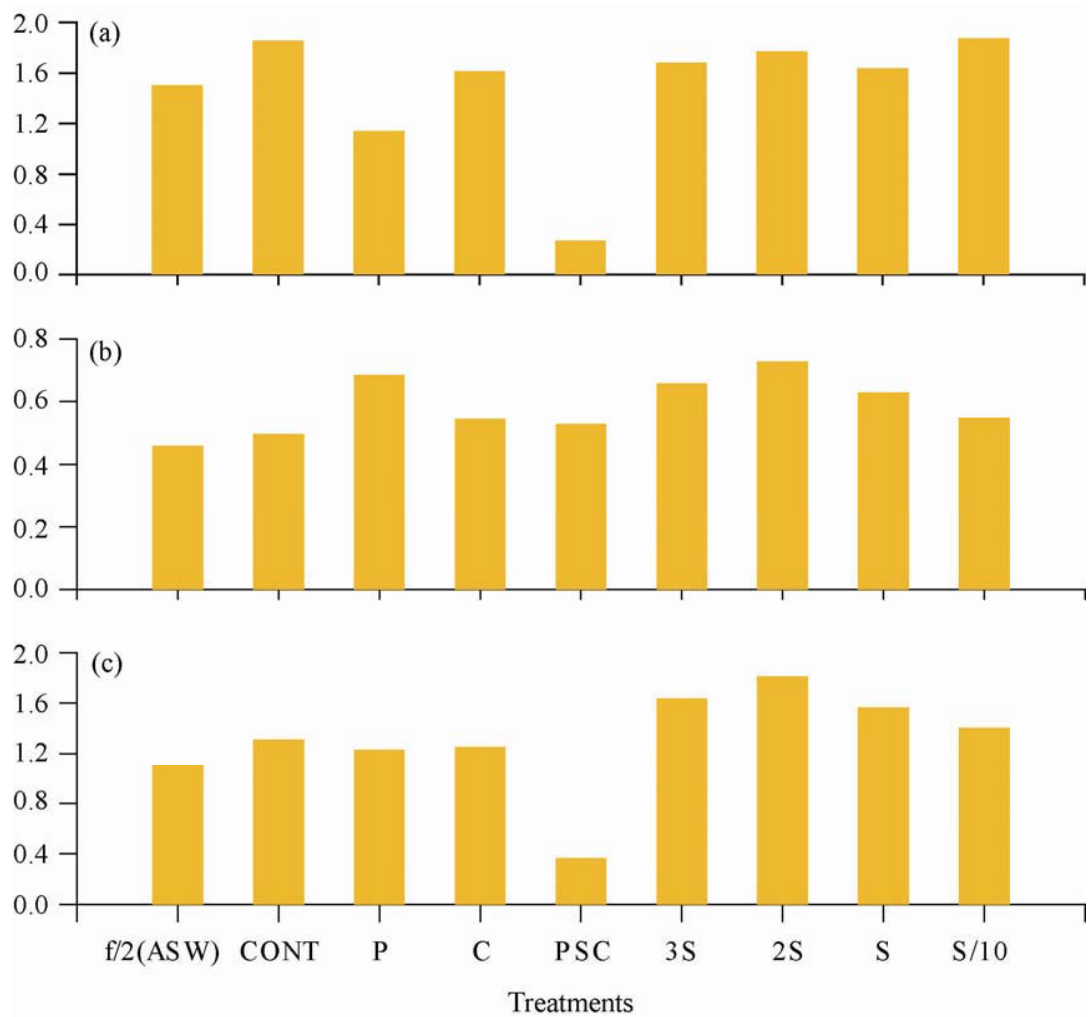
The CONT treatment supported higher average diatom abundance ( $1.16 \times 10^3$  diatoms  $\text{cm}^{-3}$  wet sediment) compared to f/2(ASW) (Fig. 3B.1a). Margalef's species richness ( $d$ ), Pielou's evenness ( $J'$ ) and Shannon-Wiener diversity ( $H'$ ) of the diatom community were also higher in CONT compared to f/2(ASW) (Fig. 3B.2). A comparison of the diatoms emerging in CONT and f/2(ASW) indicated that *Fragilariopsis* sp., *Navicula transitans* var. *derasa*, *Navicula vanhoffennii* and *Synedropsis hyperborea* were recorded only in CONT whereas *Navicula directa* was recorded only in f/2(ASW) (Table 3B.1; Fig. 3B.3). Abundance of bacteria in CONT averaged  $4.43 \times 10^9$  bacteria  $\text{g}^{-1}$  wet sediment and was similar to bacterial abundance in f/2(ASW) (Fig. 3B.1b). *V. parahaemolyticus* showed a similar trend in both CONT and f/2(ASW), except for a peak observed in CONT on day 3 (Fig. 3B.1c).



**Fig. 3B.1.** Comparison of f/2(ASW) and CONT treatments with respect to (a) diatom abundance cm<sup>-3</sup> wet sediment, (b) bacterial abundance (CFU g<sup>-1</sup> wet sediment) and (c) *Vibrio parahaemolyticus* abundance (CFU g<sup>-1</sup> wet sediment).

The penicillin treatment supported 7% diatom abundance compared to control (Fig. 3B.4a). Considering the set I treatments, diatom abundance varied significantly (Kruskal-Wallis test,  $p=0.0136$ ) and ranged from 4% in PSC to 76% in S (Fig. 3B.4a). However, a similar trend was not observed in all the streptomycin/set II treatments (Kruskal-Wallis test, ns). The set II treatments did not show a linear decrease in diatom abundance with increasing streptomycin concentration. Diatom abundance ranged from 46% (2S) to 76% (S) (Fig. 3B.4a).





**Fig. 3B.2.** Univariate measures. (a) species richness (d), (b) Pielou's evenness ( $J'$ ) and (c) Shannon-Wiener diversity ( $H'$ ) of the diatom community in the different treatments. f/2(ASW): nutrient enrichment medium (f/2) prepared in aged seawater; CONT: f/2 medium prepared in artificial seawater (MBL). Antibiotic treatment details are provided in the text.

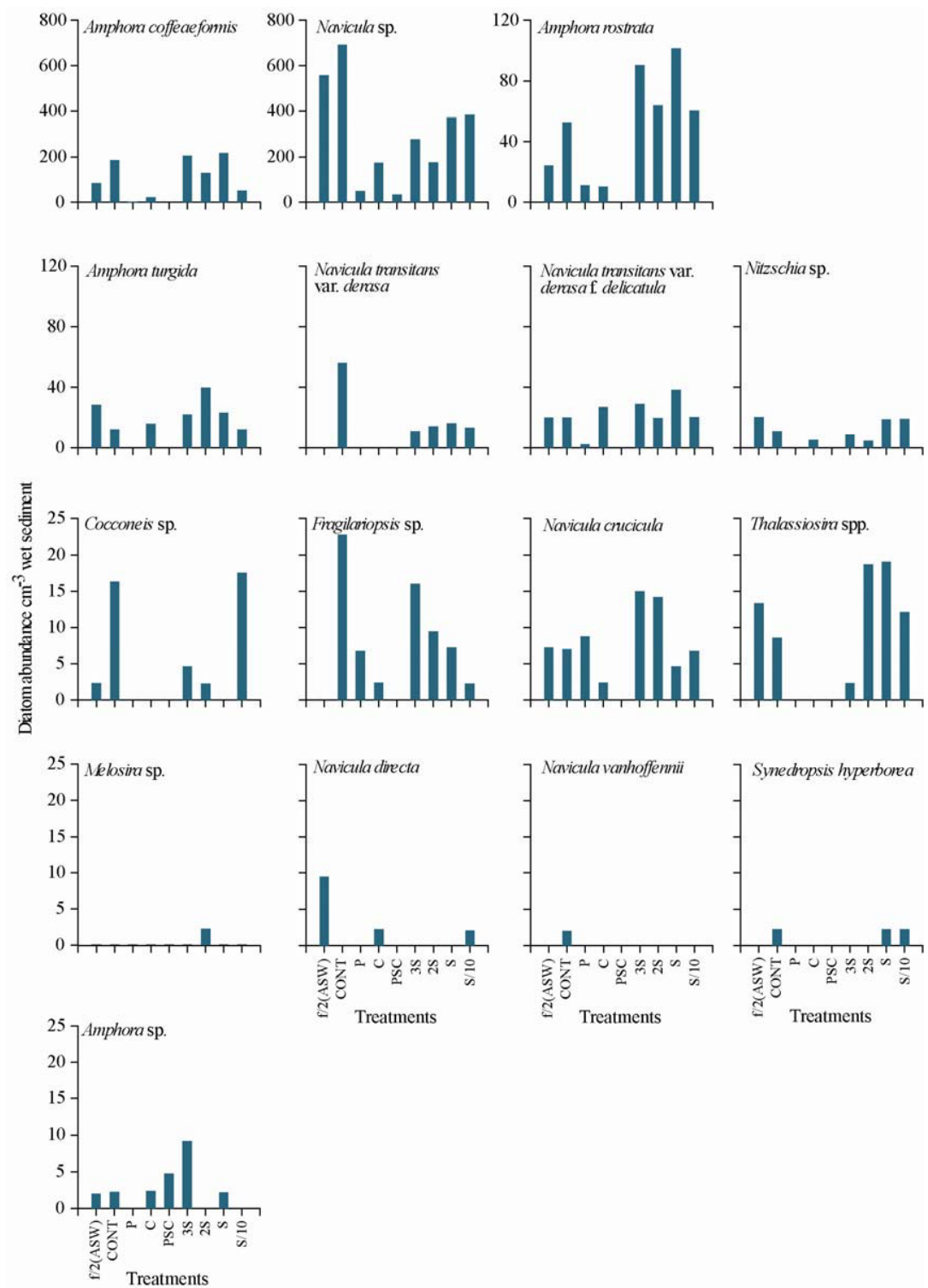
Fourteen diatom species belonging to 8 genera were recorded (Table 3B.1). *Navicula* sp. was most abundant in all the treatments (Fig. 3B.3; Table 3B.1). *Amphora rostrata*, *A. coffeaeformis*, *Fragilariopsis* sp., *Navicula crucicula* and *N. transitans* var. *derasa* f. *delicatula* were recorded in all the treatments except PSC (Fig. 3B.3). *Amphora turgida* and *Nitzschia* sp. occurred in all the treatments except P

and PSC. *N. transitans* var. *derasa* was observed in the control and all streptomycin treatments. *Cocconeis* sp. and *Thalassiosira* spp. also showed a similar distribution but were recorded in f/2(ASW) as well (Fig. 3B.3).

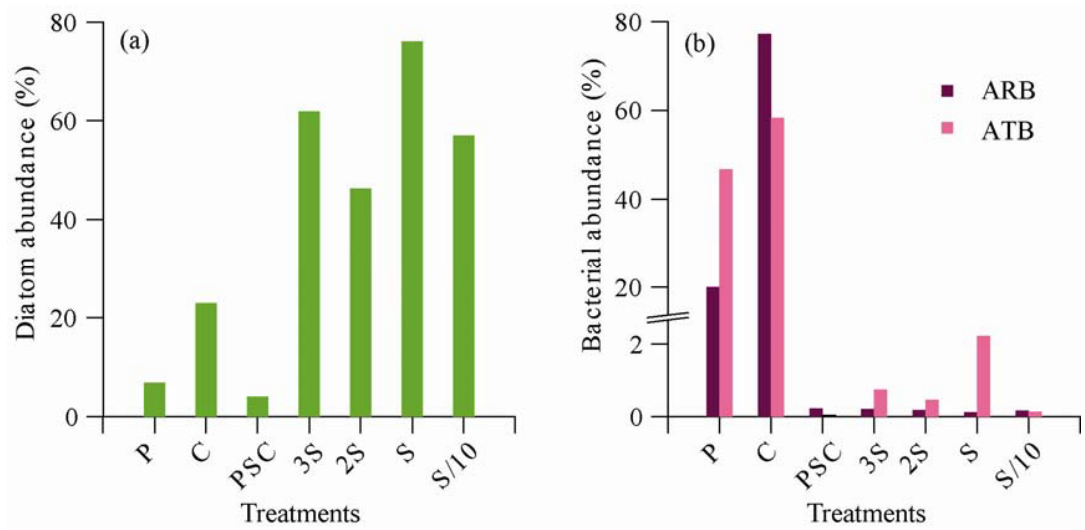
**Table 3B.1.** Diatoms observed in the different treatments. f/2(ASW): nutrient enrichment medium (f/2) prepared in aged seawater; CONT: f/2 medium prepared in artificial seawater (MBL). Antibiotic treatment details are provided in the text.

Taxon	f/2(ASW)	CONT	P	C	PSC	3S	2S	S	S/10
<i>Melosira</i> sp.							+		
<i>Thalassiosira</i> spp.	+	+				+	+	+	+
<i>Amphora coffeaeformis</i> (Agardh) Kutzing	+	+	+	+		+	+	+	+
<i>Amphora rostrata</i> Wm. Smith	+	+	+	+		+	+	+	+
<i>Amphora turgida</i> Gregory	+	+		+		+	+	+	+
<i>Amphora</i> spp.	+	+		+	+	+		+	
<i>Cocconeis</i> sp.	+	+				+	+		+
<i>Fragilariopsis</i> sp.		+	+	+		+	+	+	+
<i>Navicula crucicula</i> (Wm. Smith) Donkin	+	+	+	+		+	+	+	+
<i>Navicula directa</i> (W. Smith) Ralfs in Pritchard	+			+					+
<i>Navicula transitans</i> var. <i>derasa</i> (Grunow, in Cleve and Grunow) Cleve		+				+	+	+	+
<i>Navicula transitans</i> var. <i>derasa</i> f. <i>delicatula</i> Heimdal	+	+	+	+		+	+	+	+
<i>Navicula vanhoffenii</i> Gran		+							
<i>Navicula</i> spp.	+	+	+	+	+	+	+	+	+
<i>Nitzschia</i> spp.	+	+		+		+	+	+	+
<i>Synedropsis hyperborea</i> (Grunow) Hasle, Medlin & Syvertsen		+						+	+
Unidentified diatoms	+	+	+	+	+	+	+	+	+

The changes in diatom community structure in the antibiotic treatments were reflected in species richness, diversity and evenness measures (Fig. 3B.2). Species richness (d) in the P and PSC treatments was lower than that in CONT. Shannon-Wiener diversity (H') was lowest in PSC but higher than that in CONT in all the streptomycin treatments. Pielou's evenness (J') in all the antibiotic treatments was higher than that in control (Fig. 3B.2).



**Fig. 3B.3.** Distribution of diatoms in the different treatments. f/2(ASW): nutrient enrichment medium (f/2) prepared in aged seawater; CONT: f/2 medium prepared in artificial seawater (MBL). Antibiotic treatment details are provided in the text.



**Fig. 3B.4.** Abundance of (a) diatoms  $\text{cm}^{-3}$  wet sediment and (b) antibiotic-resistant bacteria (ARB) and antibiotic-tolerant bacteria (ATB) in the different antibiotic treatments, expressed as percentage of control values. Antibiotic treatment details are provided in the text.

Cluster analysis of all the antibiotic treatments with respect to diatoms revealed 1 group (consisting of 2 sub-groups) and 2 dissimilar entities at 50% Bray-Curtis similarity level (Fig. 3B.5a). Sub-group A consisted of all the streptomycin treatments and CONT, with abundance ranging from 538-1162 diatoms  $\text{cm}^{-3}$  wet sediment. The treatments belonging to this group were dominated by *A. coffeaeformis*, *A. rostrata* and *Navicula* sp. Sub-group B consisted of f/2(ASW) and the C treatment, having similar species distribution and with abundance ranging from 267-778 diatoms  $\text{cm}^{-3}$  wet sediment. PSC with lowest diatom abundance (48 diatoms  $\text{cm}^{-3}$  wet sediment), species richness (d) and diversity ( $H'$ ) was the most dissimilar entity. P, with 80 diatoms  $\text{cm}^{-3}$  wet sediment, was the second dissimilar treatment (Fig. 3B.5a).

### 3B.3.2. DGGE profiling, ARB and ATB

Cluster analysis of the day 7 bacterial community in all the treatments analyzed through DGGE indicated 3 groups (Fig. 3B.5b-c). Group I consisted of 2S, f/2(ASW), P and S/10; group II consisted of 3S, CONT and S, and group III consisted of C and PSC (Fig. 3B.5b-c).

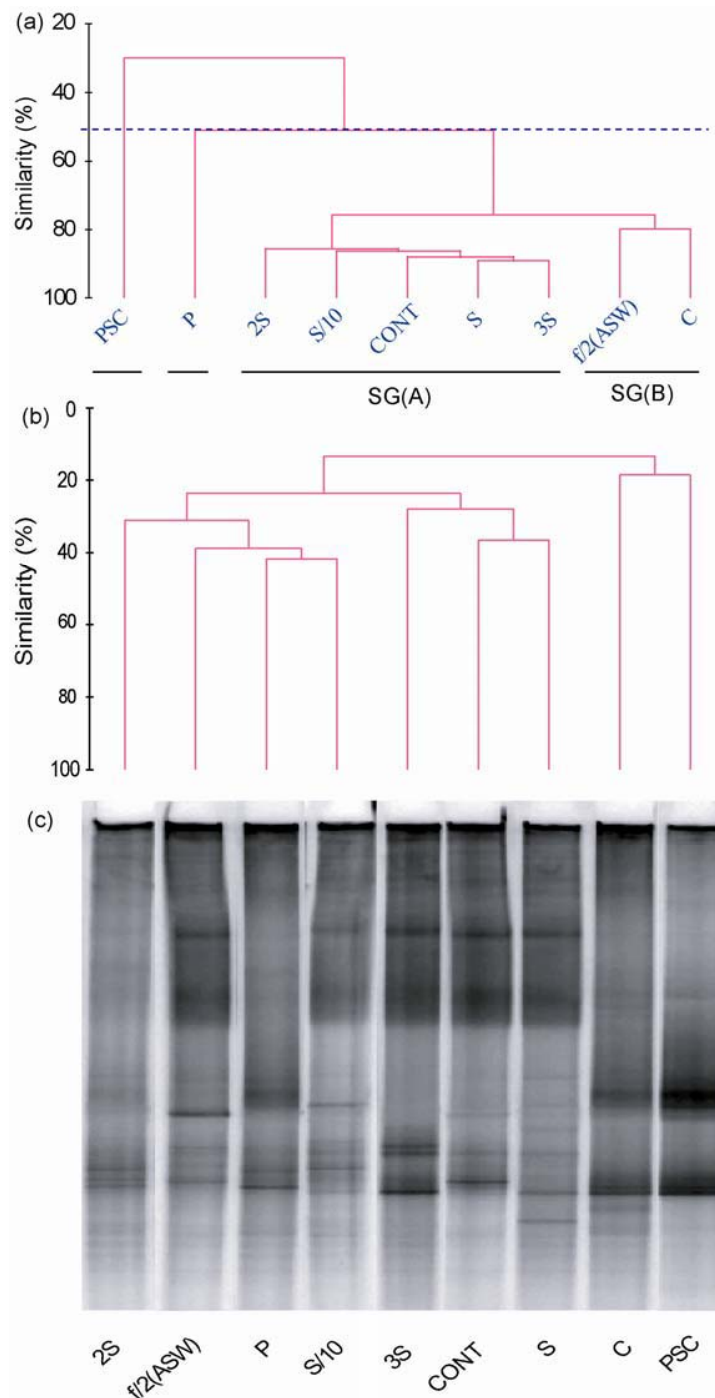
ARB and ATB varied significantly compared to bacterial abundance under control conditions, in all the antibiotic treatments, except chloramphenicol (Kruskal-Wallis test; Table 3B.2). ARB and ATB, presented as percentage values in terms of the corresponding control values, were abundant in the penicillin and chloramphenicol treatments (Fig. 3B.4b). ATB were more abundant compared to ARB in all the treatments except C, PSC and S/10 (Fig. 3B.4b).

ARB and ATB showed a lag period till day 3. In P, ARB and ATB peaked to maximum values (approx. 50%) on day 7 (Fig. 3B.6e). In C, ARB and ATB increased to 2,500% and 1000% respectively on day 4. A second peak in ARB was observed on day 6 (Fig. 3B.6f). In PSC, ARB were detected on day 4. Subsequently, both ARB and ATB increased in PSC throughout the incubation period (Fig. 3B.6g).

Considering the set II treatments, ARB and ATB were <10% of the corresponding control values in 3S, 2S and S and comparatively higher in S/10 (Fig. 3B.6a-d). ARB and ATB were maximum on day 4 (Fig. 3B.6a-d).

### 3B.3.3. Dynamics of *Vibrio parahaemolyticus*

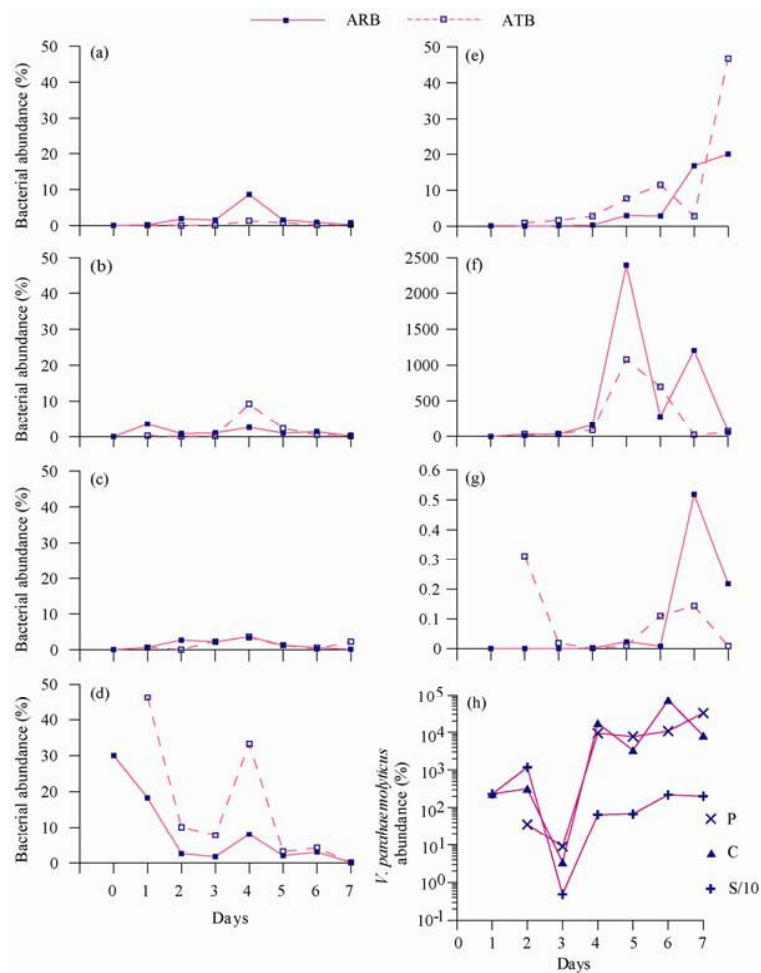
*V. parahaemolyticus* were sensitive to the antibiotic combination (PSC) and higher streptomycin concentrations (3S, 2S and S). In fact, *V. parahaemolyticus* abundance



**Fig. 3B.5.** Cluster dendrogram of the different treatments using Bray-Curtis similarity, based on the (a) diatom community, (b) the bacterial community DGGE profile (c). f/2(ASW): nutrient enrichment medium (f/2) prepared in aged seawater; CONT: f/2 medium prepared in artificial seawater (MBL). Antibiotic treatment details are provided in the text. Dashed line (----) in (a) indicates grouping at 50% similarity. SG(A): Sub-group A; SG(B): Sub-group B.

**Table 3B.2.** Significance values of the Kruskal-Wallis tests for abundance of ARB, ATB and *V. parahaemolyticus* in antibiotic treatments compared to control conditions. Antibiotic treatment details are provided in the text. Values in bold indicate significant variation.

Antibiotic treatments	p-value		
	ARB	ATB	<i>V. parahaemolyticus</i>
P	<b>0.0007</b>	<b>0.0038</b>	0.2126
C	0.2507	0.6133	0.1068
PSC	<b>0.00001</b>	<b>0.00001</b>	<b>0.00002</b>
3S	<b>0.00002</b>	<b>0.00001</b>	<b>0.00002</b>
2S	<b>0.00001</b>	<b>0.00002</b>	<b>0.00002</b>
S	<b>0.00001</b>	<b>0.00002</b>	<b>0.00002</b>
S/10	<b>0.00002</b>	<b>0.0001</b>	0.3558



**Fig. 3B.6.** Abundance of ARB and ATB in (a) 3S, (b) 2S, (c) S, (d) S/10, (e) P, (f) C and (g) PSC, and (h) *Vibrio parahaemolyticus* in the P, C and S/10 treatments, throughout the incubation period, expressed as percentage of respective control values. Antibiotic treatment details are provided in the text.

varied significantly compared to control conditions, in only these particular antibiotic treatments (Kruskal-Wallis test; Table 3B.2). They occurred in S/10, at 199% of the corresponding control values and multiplied to very high numbers in the P and C treatments (Fig. 3B.6h).

### **3B.4. Discussion**

The CONT diluent supported higher diatom abundance compared to f/2(ASW) and similar bacterial profiles with the exception of the *V. parahaemolyticus* peak on day 3. Incorporation of penicillin in the control diluent resulted in the most dissimilar diatom community among all the individual antibiotic treatments.

Penicillin cannot affect diatoms directly as it inhibits peptidoglycan synthesis mainly in Gram positive bacteria and diatoms have a different cell wall structure (Tomas 1997). Yet, diatom abundance was reduced to 7% (Fig. 3B.4a). Besides, species richness in the penicillin treatment was the lowest among all the individual antibiotic treatments, highlighting the relevance of bacteria to diatoms.

The subset of bacteria inhibited by penicillin and consisting mainly of Gram positive bacteria, appears to be important for the growth of *A. coffeaeformis*, one of the dominant diatoms in the study area (Mitbavkar and Anil 2002, 2006). It occurred in low abundance in the penicillin treatment (Table 3B.1; Fig. 3B.3). *A. coffeaeformis* is a fast reproducer and has strong competitive potential (Mitbavkar and Anil 2007). It produces a water-soluble toxin (Maranda et al. 1989) and is reported to have toxic effects (Buzzelli et al. 1997). It can overtake *N. transitans* var. *derasa* f. *delicatula* even when present at a ratio of 1:99 (Mitbavkar and Anil 2007). Earlier studies have revealed that bacteria are important to *A. coffeaeformis*. Firstly,



axenic cultures of *A. coffeaeformis* have a different EPS composition compared to non-axenic cultures (Patil and Anil 2005). Secondly, axenic cultures display enhanced sensitivity to metals (Cu and tributyltin) than non-axenic cultures (Thomas and Robinson 1987). This work add another dimension to these studies, i.e., they reiterate the relevance of penicillin-sensitive bacteria in influencing the population dynamics of this otherwise competitive diatom.

The penicillin treatment was most similar to the PSC treatment. This was evident not only in the cluster analysis, but also in the species occurrence profiles (Table 3B.1; Fig. 3B.5a). Diatoms were most sensitive to PSC, and were suppressed to 4% (Fig. 3B.4a) whereas Margalef's species richness and Shannon-Wiener diversity were also low (Fig. 3B.2). *A. coffeaeformis*, *A. rostrata*, *Fragilariopsis* sp., *Navicula crucicula* and *N. transitans* var. *derasa* f. *delicatula* that were observed in all the individual antibiotic treatments, were noticeably absent in PSC (Table 3B.1; Fig. 3B.3). The inhibition of most diatoms in PSC could be attributed to the combined effects of all the 3 antibiotics used. Penicillin inhibits peptidoglycan synthesis during cell wall formation; it is active mainly against Gram-positive bacteria. Streptomycin affects mainly Gram-negative bacteria (by binding to the 30S ribosomal subunit) (Josephson et al. 2000). Chloramphenicol, a known inhibitor of photosynthesis in diatoms (Ohad et al. 1984), inhibits both Gram-positive and Gram-negative bacteria, by binding to the 50S ribosomal subunit (Josephson et al. 2000). Therefore, the combination of all 3 antibiotics affects a wider spectrum (analogous to functional types) of bacteria than individual antibiotics alone.

This was reflected in the detection of PSC-resistant bacteria only on day 4, reflecting a period of adaptation to the combined presence of the 3 antibiotics. The

appearance of PSC-resistant bacteria stabilized the bacterial community; both PSC-resistant and PSC-tolerant bacteria increased subsequently. However, on day 7, the abundance of both groups was still suppressed compared to day 7 control values (Fig. 3B.4b), indicating the continued effectiveness of the antibiotic combination.

Marked changes in diatom abundance were also observed in the other Set I treatments. Streptomycin and chloramphenicol, though having the potential to affect diatoms directly, affected diatoms differentially (Fig. 3B.4a). Diatom abundance was reduced to 23% in the chloramphenicol treatment (Fig. 3B.4a). An interesting trend in the abundance profiles of both chloramphenicol-resistant and chloramphenicol-tolerant bacteria was noticed. Both these groups of bacteria increased explosively after the 3 day lag period but decreased to 77% (chloramphenicol-resistant bacteria) and 58% (chloramphenicol-tolerant bacteria) of control values on day 7 (Fig. 3B.6f). Probably, the inhibition of bacteria by chloramphenicol relaxed the growth constraints (biotic constraints; decreases competition by other bacteria) on both groups of bacteria in the initial stages of growth. However, this increase was not sustained till the end of the incubation period. Such changes in bacterial dynamics during the incubation period will impact the emerging diatom community.

Though growth of most diatoms was reduced in chloramphenicol, they did not seem to be significantly inhibited by streptomycin. A dose-dependent response of the diatom community to different streptomycin concentrations was not observed (Fig. 3B.4a). In fact, *Amphora* and *Navicula* species occurred abundantly in the streptomycin treatments, in some instances at levels far higher than that in control (Fig. 3B.3), whereas other species, namely *Cocconeis* sp., *N. transitans* var. *derasa* and *Thalassiosira* spp. were observed in only the streptomycin treatments among all

the antibiotic treatments (Table. 3B.1). All the streptomycin treatments were most similar to the control diluent (Fig. 3B.5) with no significant variation observed (Kruskal-Wallis test; ns). This similarity was also evident in the species richness, evenness and diversity indices of the diatom community in the control and streptomycin treatments (Fig. 3B.2). Though diatoms did not seem to be inhibited by streptomycin, both streptomycin-resistant and streptomycin tolerant bacteria were suppressed to <10% in 3S, 2S, S and S/10, with the exception of streptomycin tolerant bacteria in S/10 (approximately 35%) (Fig. 3B.4b). This disparity in the abundance profiles of diatoms and bacteria suggest that the particular subset of bacteria suppressed by streptomycin were not essential for diatom growth.

To further understand the changes in diatom community structure, it is important to know the changes in bacterial dynamics that the multiplying diatom community is exposed to throughout the incubation period. ARB were more abundant than ATB in the C and PSC treatments (Fig. 3B.4b), indicating the preference for antibiotic resistance over antibiotic tolerance. This is evident in the grouping of C and PSC when subjected to cluster analysis of the bacterial community based on DGGE band profiles (Fig. 3B.5b-c). It however differed from the cluster analysis of the treatments based on diatoms (Fig. 3B.5a), which indicated that the C and f/2(ASW) treatments supported similar diatom communities. These differences in cluster analyses patterns of diatom and bacterial communities need to be evaluated in detail.

Among the individual antibiotics, penicillin affects diatom community structure to a maximum extent; this effect could be due to the changes in penicillin-resistant and penicillin-tolerant bacteria. ATB were more abundant than ARB in the penicillin treatment (45% and 20% respectively) (Fig. 3B.4b). ATB represent a pool of future

antibiotic resistant bacteria, characterized by unknown/hidden functional types. The ARBs have already passed through a genetic bottleneck, and thus have reduced functional diversity, so-called loss of fitness (Anderson 2005). The strategy of ATBs, that of surviving in the non-multiplying state, helps bacteria to survive in the presence of antibiotics that act on growing cells, for e.g.,  $\beta$ -lactams such as penicillin (Miller et al. 2004). Antibiotic tolerance may be part of a highly conserved and generalized mechanism for bacteria to tolerate environmental stress (Harrison et al. 2005), and increases chances of survival of bacterial populations in fluctuating environments (Balaban et al. 2004). Characteristic shifts in the dynamics of ARB and ATB have also been observed in the other antibiotic treatments (Fig. 3B.6). These shifts in ARB and ATB dynamics, whether mediated by loss of transport or regulatory proteins, are most probably associated with a change in the physiological status of bacteria. For e.g., in *E. coli*, resistance to streptomycin may dramatically reduce the rate of protein biosynthesis (Worden et al. 2006), thereby representing an example of loss-of-fitness (Anderson 2005). These changes are indicative of the physiological/functional shifts in bacterial communities when treated with antibiotics. Additionally, the results observed with penicillin reveal that any change in the bacterial community (functional types) due to a shift in the dynamics between penicillin-resistant and penicillin-tolerant bacteria, will be reflected in changes in the diatom community structure. This has important implications from the point of view of changes in bacterial communities being ultimately reflected in diatom communities.

*V. parahaemolyticus*, that constituted <0.1% of the culturable bacterial community in sediment (data not shown), were sensitive to the antibiotic combination (PSC) and 3S, 2S and S treatments but multiplied to very high numbers in P and C (Fig. 3B.6h).

Probably, *V. parahaemolyticus* responded to relaxation of growth constraints/competitive forces (through inhibition of certain subgroups of bacteria by P and C) by multiplying rapidly. It is also possible that *V. parahaemolyticus* emerged from the VNC state and proliferated when competing bacteria were inhibited by antibiotics. However, the sensitivity of *V. parahaemolyticus* to 3S, 2S and S in addition to its lowered abundance in S/10 (Fig. 3B.6h), when compared to changes in diatom community structure, indicate that these bacteria are not essential for diatom growth.

Summarizing the results, microphytobenthic diatom communities, when treated with penicillin, showed marked reduction in species richness and diatom abundance (especially *A. coffeaeformis*, one of the dominant diatoms in the study area). These changes occurred in response to the variations in dynamics of penicillin-resistant and -tolerant bacteria and were different from the potentially direct effects of streptomycin and chloramphenicol (as indicated even in the DGGE profile). These observations, along with the shift in *V. parahaemolyticus* dynamics throughout the incubation period, illustrate the range of interactions between specific bacterial subsets and diatoms, and their significance as an important factor regulating diatom communities in benthic environments.

### ***3C. Effect of antibiotics on diatom monocultures isolated from the study area***

#### ***3C.1. Introduction***

The ability of bacteria to modulate diatoms at the community level was investigated using the antibiotics approach. But how are diatoms affected by antibiotics when in monoculture?

In nature, diatoms usually occur in multi-species communities, and their physiology is markedly different from diatoms under culture conditions. These differences are also apparent in the number of bacteria attached to the exterior surfaces of diatoms. The number of bacteria on diatom surfaces is far higher under culture conditions, compared to natural conditions (Kaczmarska et al. 2005). This is especially relevant in the context of the interdependence of diatoms and bacteria. Diatoms rely on bacteria for vitamins (Croft et al. 2005, Droop 2007) whereas bacteria require extracellular diatom metabolites for survival (Reimann et al. 2000, Lau et al. 2007).

In view of these close associations between diatoms and bacteria, the following questions were addressed. (1) What is the effect of antibiotics on diatom monocultures isolated from the study area? (2) How does antibiotic treatment affect the bacterial community in diatom monocultures?

### **3C.2. Materials and methods**

#### *3C.2.1. The effect of antibiotics on diatom monocultures*

The effect of antibiotics on diatoms was assessed using near-axenic cultures of *Amphora coffeaeformis*, *Coscinodiscus* sp., *Skeletonema costatum* and *Thalassiosira* sp., isolated from the study area. *A. coffeaeformis* is a dominant pennate diatom in the study area whereas *Coscinodiscus* sp. and *Thalassiosira* sp. (centric diatoms), each contributing <5%, are minor components of the diatom community. *S. costatum*, is an important blooming diatom in the waters surrounding the intertidal sandflat but was not encountered in this study. All the diatom cultures were maintained in aged seawater enriched with f/2 level of nutrients (Guillard and Ryther 1962) [f/2(ASW)], and incubated under a 12h: 12h light: dark cycle. They were subcultured into fresh medium every 7 days and checked microscopically at regular intervals. Prior to the experiment, they were transferred into f/2(MBL) and subcultured twice.

The diatom cultures were subjected to a combination of physical and chemical methods to render them axenic (Patil and Anil 2005). The diatom cultures were repeatedly washed (10 times) with sterile f/2(MBL) before and after mild sonication to remove loosely adhered bacterial cells. This was followed by overnight incubation in f/2(MBL) containing 1mg mL<sup>-1</sup> lysozyme to eliminate Gram-negative bacteria. The cultures were washed once again (10 cycles), checked microscopically to detect cell wall permeabilization, and incubated overnight in f/2(MBL) containing a mixture of penicillin G (1 mg mL<sup>-1</sup>), streptomycin (0.5 mg mL<sup>-1</sup>) and chloramphenicol (0.2

mg mL<sup>-1</sup>). The cultures were then transferred to antibiotic-free medium. *S. costatum* lost viability following this treatment. Axenic status of the cultures was determined through fluorochrome staining with DAPI and spread plating on ZMA 2216. Bacteria were reduced below detectable levels in *A. coffeaeformis* and by approximately 99% in *Coscinodiscus* sp. and *Thalassiosira* sp. The cultures were then incubated in antibiotic-free medium for 6 days. Subsequently, bacteria were detected at low levels (<0.01% of non-axenic culture values) in *A. coffeaeformis*. However, considering the loss of viability of diatoms when treated with slightly higher antibiotic concentrations, these ‘near-axenic’ diatom cultures were used to determine the effect of antibiotics on diatoms.

The diatom cultures were transferred to f/2(MBL) containing penicillin, streptomycin and chloramphenicol, individually and in combination, and at various concentrations (Fig. 3A.2 on page 61). This led to different levels of suppression of the residual bacteria in the diatom cultures. A control [f/2(MBL)] was also maintained to allow the residual bacteria to grow unhindered. *A. coffeaeformis* and *Thalassiosira* sp. were inoculated at 100 cells mL<sup>-1</sup> initial concentration and *Coscinodiscus* sp. at 25 cells mL<sup>-1</sup>. Triplicates were maintained for each treatment. After incubation for 7 days, viable diatoms were enumerated using a combination of light microscopy and autofluorescence. Bacteria were also enumerated on ZMA plates, after incubation at room temperature for 48 hrs.



### 3C.2.2. The effect of penicillin on the bacterial community in diatom monocultures

*A. coffeaeformis*, *Coscinodiscus* sp., *S. costatum* and *Thalassiosira* sp.), isolated from the study area, were used for this analysis. The diatom cultures were maintained in f/2(MBL) and subcultured thrice. During the exponential phase, appropriate aliquots of the culture (in triplicate) were withdrawn for quantification and isolation of free-living and diatom-associated bacteria. Free-living bacteria (FLB) were isolated by spread plating appropriate aliquots on Zobell Marine Agar (ZMA) 2216, followed by incubation at room temperature for 48 hrs. Diatom-associated bacteria (DAB) were isolated following the method by Kaczmarek et al. (2005). Two mL of the culture was filtered through a 3µm polycarbonate filter using a sterile vacuum filtration apparatus. The filter paper was rinsed with 0.22 µm filtered and autoclaved seawater, and inverted onto ZMA 2216 plates. After incubation for 48 hrs at room temperature, the visible colonies were quantified and purified by repeated streaking on ZMA 2216 plates and subsequently maintained on ZMA 2216 slants at 5°C. The bacterial isolates were identified to genus level based on morphological and biochemical tests following Bergey's Manual of Systematic Bacteriology (Holt et al. 1994).

To determine the effect of penicillin on the bacterial community in diatom monocultures, the diatom cultures were transferred to f/2(MBL) containing 0.2 mg mL<sup>-1</sup> penicillin, and incubated for 7 days at 25±1 °C with a 12 h:12 h light : dark cycle. The FLB<sub>p</sub> (FLB after penicillin treatment) were quantified, isolated on ZMA 2216 medium and identified using Bergey's Manual of Systematic Bacteriology (Holt et al. 1994).

### 3.C.2.3. *The antibacterial activity of diatom-associated bacteria*

The antibacterial activity of 6 DAB isolates was assayed (in triplicate) using the agar plug method (Hentschel et al. 2001). A total of 15 bacteria, FLB and DAB (details in Table 3C.1), were used as test strains. The DAB to be tested for antibacterial activity were grown overnight as a lawn on Zobell marine agar, by plating out 150  $\mu$ l on ZMA 2216 plates. Plugs of 10 mm diameter were stanced out with a cork-borer and placed with the bacterial side down on agar plates, which had been seeded with a stationary phase culture of the test bacterial strain. Agar plugs without bacteria were used as controls and were observed to not inhibit bacterial growth. The diameter of the inhibition zone surrounding the agar plugs was measured after 48 h incubation at 30°C. Results are presented in the form of percentage inhibition of test bacterial isolates (number of bacteria inhibited/total number of bacteria tested in the assay  $\times$  100).

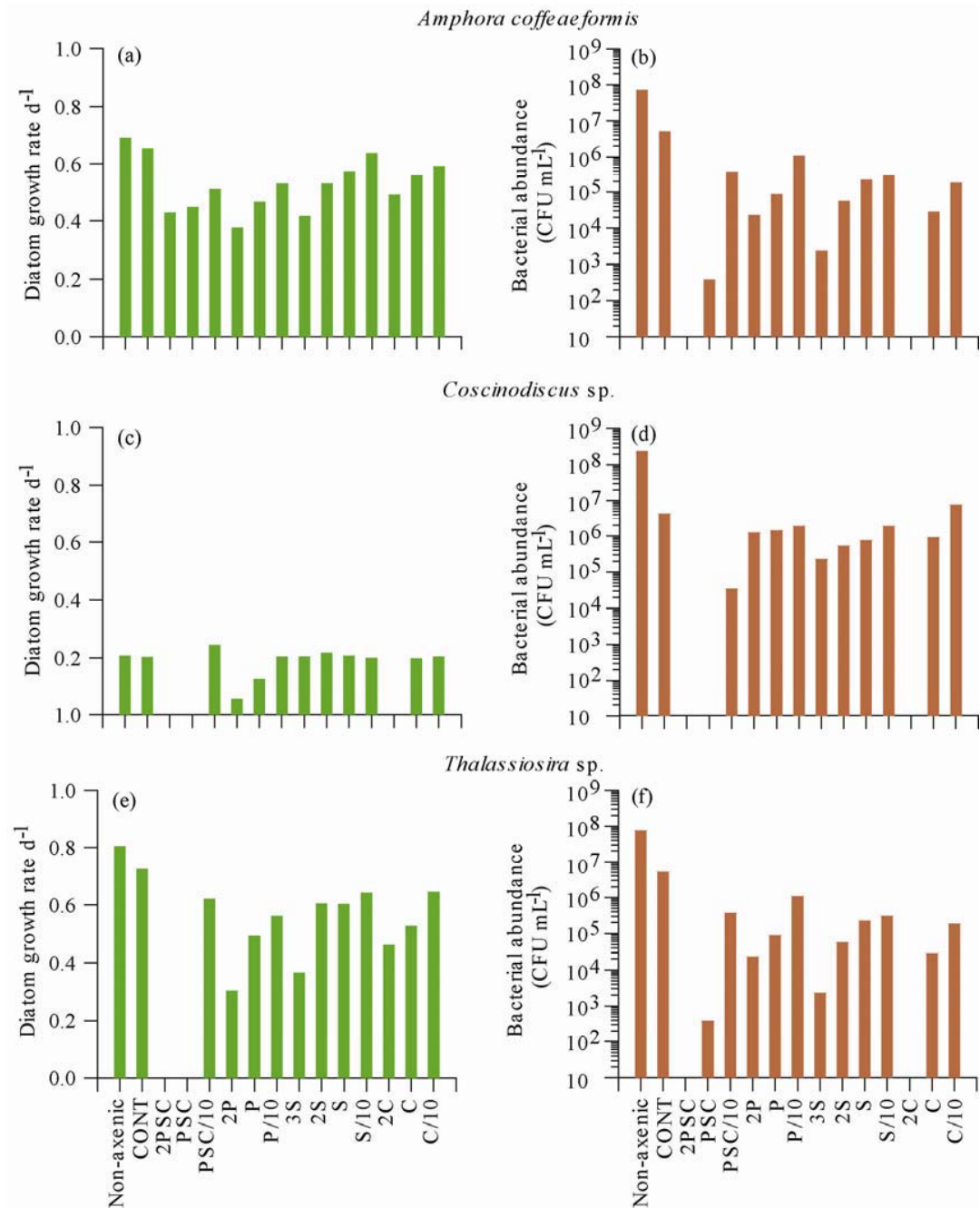
### 3C.2.4. *Data analysis*

Growth rate  $\text{day}^{-1}$  of near-axenic *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp. were calculated using the formula, growth rate =  $(\log_{10} N - \log_{10} N_0) * 2.3 / t$ , where N = cell concentration at the end of the incubation period and  $N_0$  = cell concentration at  $t = 0$  (day 0).

## 3C.3. *Results*

### 3C.3.1. *The effect of antibiotics on diatom monocultures*

Growth rates of the near-axenic diatoms were lower in most antibiotic treatments compared to control values (Fig. 3C.1a,c,e). The 2PSC and PSC treatments did not

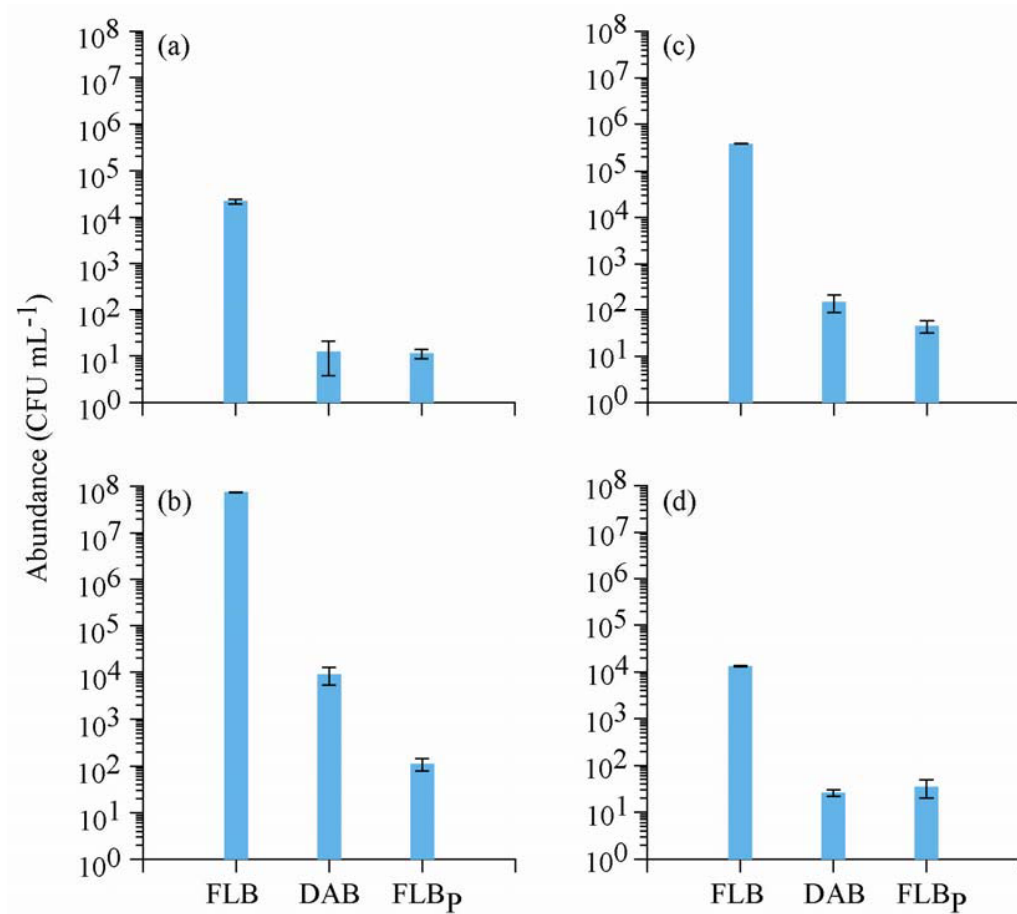


**Fig. 3C.1.** Growth rate of diatoms (a,c,e) and bacterial abundance (mean values) (b,d,f) in (a,b) *Amphora coffeaeformis*, (c,d) *Coscinodiscus* sp. and (e-f) *Thalassiosira* sp. monocultures in the different treatments. Antibiotic treatment details are provided in Fig. 3A.2.

support growth of *Coscinodiscus* sp. and *Thalassiosira* sp.; however, *A. coffeaeformis* survived in these treatments. The penicillin treatments supported growth of *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp., but at lower growth rates. A similar trend was observed in the chloramphenicol treatments except for *Coscinodiscus* sp. that did not grow in the 2C treatment (Fig. 3C.1a,c,e). An erratic trend in growth rates, especially of *Coscinodiscus* sp. was observed in the streptomycin treatments (Fig. 3C.1a,c,e). Bacteria in near-axenic *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp. recovered to approximately 7% of bacterial abundance values in non-axenic diatom cultures (Fig. 3C.1b,d,f). Bacteria were suppressed to below control values in all the 3 cultures in the different antibiotic treatments with the exception of *Coscinodiscus* sp. in the C/10 treatment (Fig. 3C.1d).

### 3C.3.2. The effect of penicillin on the bacterial community in diatom monocultures

FLB and DAB were highest in the *Coscinodiscus* sp. monoculture (Fig. 3C.2). FLB ranged from  $1.32 \times 10^4$  CFU mL<sup>-1</sup> -  $7.47 \times 10^7$  CFU mL<sup>-1</sup> whereas DAB ranged from  $1.2 \times 10^1$  CFU mL<sup>-1</sup> -  $9 \times 10^3$  CFU mL<sup>-1</sup> (Fig. 3C.2). The dominant FLB were identified as *Alteromonas*, *Bacillus*, *Microbacterium*, *Micrococcus* and *Pseudomonas* (Table 3C.1). DAB isolates from *Amphora coffeaeformis* lost viability on subculturing. The DAB isolates from *Coscinodiscus* sp. and *S. costatum* were similar to the respective FLB fractions. However, the DAB isolates from *Thalassiosira* sp. differed from the FLB fraction and were identified as *Alteromonas* and *Micrococcus* (Table 3C.1).



**Fig. 3C.2.** Abundance of FLB, DAB and FLB<sub>p</sub> fractions of bacteria in monocultures of (a) *Amphora coffeaeformis*, (b) *Coscinodiscus* sp. (c) *Thalassionema* sp. and (d) *Skeletonema costatum*.

Treatment with penicillin led to a decrease in FLB, from  $1.1 \times 10^1$  CFU mL<sup>-1</sup> in *A. coffeaeformis* to  $1.1 \times 10^2$  CFU mL<sup>-1</sup> in *Coscinodiscus* sp. (Fig. 3C.2). A noticeable change in the FLB community in the *S. costatum* was observed, i.e., *Flavobacterium* dominated after penicillin treatment (Table 3C.1).

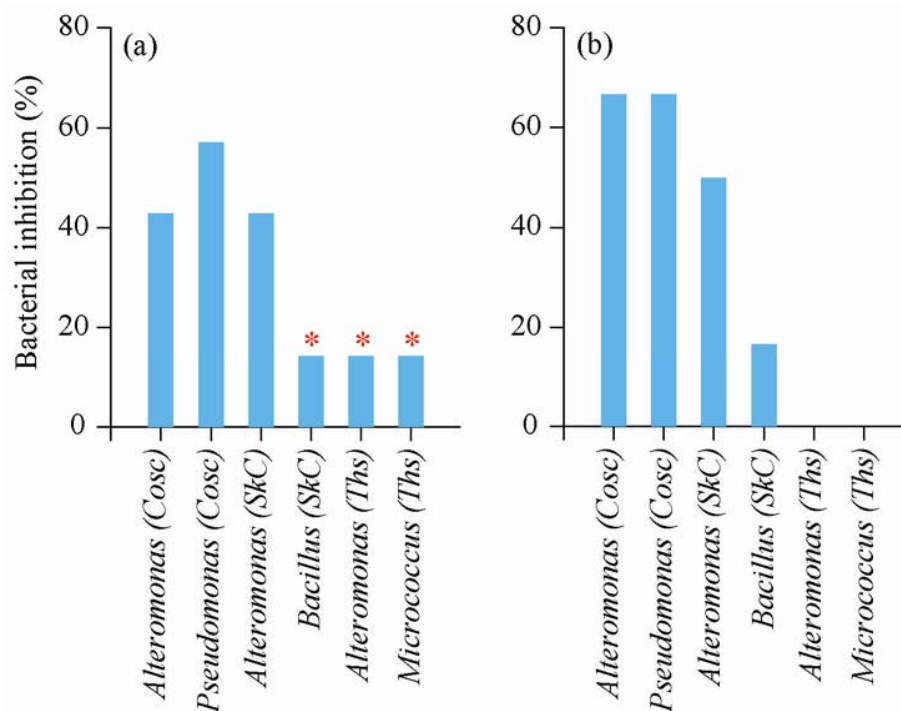
**Table 3C.1.** Details of free-living and diatom-associated bacterial fractions and post-penicillin treated bacterial fractions isolated from diatom monocultures.

Diatom monocultures	Bacteria		
	Free-living (FLB)	Diatom-associated (DAB)	Post-penicillin treatment (FLB <sub>p</sub> )
<i>Amphora coffeaeformis</i>	<i>Alteromonas</i> <i>Micrococcus</i>	*	*
<i>Coscinodiscus</i>	<i>Alteromonas</i> <i>Pseudomonas</i>	<i>Alteromonas</i> <i>Pseudomonas</i>	<i>Alteromonas</i> <i>Micrococcus</i>
<i>Skeletonema costatum</i>	<i>Alteromonas</i> <i>Bacillus</i>	<i>Alteromonas</i> <i>Bacillus</i>	<i>Flavobacterium</i>
<i>Thalassiosira</i>	<i>Bacillus</i> <i>Microbacterium</i> <i>Pseudomonas</i>	<i>Alteromonas</i> <i>Micrococcus</i>	<i>Alteromonas</i> <i>Flavobacterium</i>

\* lost viability on subculturing

### 3.C.3.3. The antibacterial activity of the diatom-associated bacteria

The isolates from *Coscinodiscus* sp. and *S. costatum* showed antibacterial activity, with zones of inhibition from 11-17 mm (Table 3C.2). Their antibacterial activity ranged from 14-57% against FLB and 17-67% against DAB, with highest values observed in the isolates from *Coscinodiscus* sp. (Fig. 3C.3). Both DAB isolates from *Thalassiosira* sp., in addition to 1 DAB from *S. costatum* (*Bacillus* sp.), stimulated the growth of 1 FLB isolate from *Coscinodiscus* sp., with zones of stimulation ranging from 22-25 mm (Table 3C.2).



**Fig. 3C.3.** The bacterial inhibition (%) of the DAB isolates against (a) FLB fractions and (b) DAB fractions. \* indicates stimulation of bacterial growth.

### 3C.4. Discussion

The differential effect of antibiotics on diatoms was also evident in growth rate profiles of near-axenic diatom monocultures grown in presence of antibiotics. Near-axenic diatom cultures were used as controls as repeated attempts to obtain axenic cultures were unsuccessful. Probably, some bacteria are hidden in spaces in the diatom frustules, in between cingulae, where they are not accessible to antibiotics and other antimicrobial agents, and are also not dislodged by rinsing procedures (Kaczmariska et al. 2005). The growth rates of these near-axenic diatoms in antibiotic-free medium were slightly lower than that of non-axenic diatoms with the exception of *Coscinodiscus* sp. in the non-penicillin treatments (Fig. 3C.1a,c,e).

**Table 3C.2.** Antibacterial activity of DAB isolates.

	Diameter of inhibition zone (mm)					
	Tested DAB isolates					
	<i>Alteromonas</i> (Cos)	<i>Pseudomonas</i> (Cos)	<i>Alteromonas</i> (SkC)	<i>Bacillus</i> (SkC)	<i>Alteromonas</i> (Thss)	<i>Micrococcus</i> (Thss)
<b>Free-living bacteria (FLB)</b>						
<i>Alteromonas</i> (SkC)	11	13				
<i>Bacillus</i> (SkC)	11	12				
<i>Bacillus</i> (Thss)			16	13		
<i>Microbacterium</i> (Thss)	12	12	13			
<i>Pseudomonas</i> (Thss)		13				
<i>Alteromonas</i> (Cos)						
<i>Pseudomonas</i> (Cos)			12	<b>22</b>	<b>22</b>	<b>25</b>
<b>Diatom-associated bacteria (DAB)</b>						
<i>Alteromonas</i> (SkC)	14	17				
<i>Bacillus</i> (SkC)	12	13				
<i>Alteromonas</i> (Thss)	11	13	12			
<i>Micrococcus</i> (Thss)	11	13	13			
<i>Alteromonas</i> (Cos)						
<i>Pseudomonas</i> (Cos)			14	13		

**red font** indicates stimulation of bacterial growth

The similarity in growth rates could be attributed to 2 reasons. Firstly, bacteria may not have had any effect on diatom growth. Secondly, the near-axenic cultures may have recovered their original bacterial population from the residual seed bacteria present in the diatom cultures, when grown in antibiotic-free medium (Fig. 3C.1b,d,f). In the present study, bacteria in the near-axenic controls recovered to approximately 7% of bacterial abundance values in the non-axenic controls (Fig.



3C.1b,d,f). Recovery of bacteria in near-axenic diatom cultures when grown in antibiotic-free medium is one of the problems associated with obtaining and maintaining axenic diatom cultures (Kaczmarska et al. 2005).

When recovery of the bacterial population was suppressed in the different antibiotic treatments, growth rates of *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp. were comparatively lower in all the antibiotic treatments, except for *Coscinodiscus* sp. in the non-penicillin treatments, indicating species-specific responses to antibiotics. This aspect is important in view of these species-specific responses being reflected in other benthic diatoms as well. Among the individual antibiotic treatments, streptomycin seemed to be the least effective in inhibiting diatoms and showed an erratic enhancement/inhibition trend for *Coscinodiscus* sp. This is similar to the trend observed in the experiments with natural diatom communities from sediment (Chapter 3B). The penicillin treatments supported growth of *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp., but at lower growth rates, revealing the importance of bacteria to these diatoms. This differs from the results of the experiments with natural diatom communities, wherein these 3 diatoms were not observed in the high (2P) and moderate (P) penicillin treatments (Table 3A.2, Fig. 3C.1a,c,e). A similar difference was observed in the distribution of *Thalassiosira* sp. in the chloramphenicol treatments (Table 3A.2, Fig. 3A.8). This discrepancy in distribution patterns indicates the difference in survival characteristics between monoculture and multiculture conditions, and the relevance of the initial inoculum conditions (Mitbavkar and Anil 2007).

Among the diatom monocultures analyzed, the DAB isolates from *A. coffeaeformis* lost viability; they probably grow only in close contact with *A.*

*coffeaeformis* cells. *Coscinodiscus* sp. had the lowest growth rates, under control conditions as well in the antibiotic treatments (Fig. 3C.1). The *Coscinodiscus* sp. monoculture supported the highest FLB, DAB and FLB<sub>p</sub> fractions (Fig. 3C.2). Additionally, the DAB isolates from *Coscinodiscus* sp. had the highest antibacterial activity against both, FLB and DAB fractions (Fig. 3C.3). These observations point to the species-specific associations of bacteria with diatom monocultures. Such species-specific associations have been observed earlier in *Coscinodiscus* sp. and other diatoms (Schafer et al. 2002).

Given that the associations of bacteria with diatom monocultures are specific, the changes in the FLB fraction when treated with penicillin are significant. The most noticeable change was observed in *S. costatum*. This shift in the bacterial community in *S. costatum* monocultures when treated with penicillin is important mainly because the DAB isolates from *Skeletonema* have a wide range of antibacterial activity (ranging from inhibition to stimulation) against both FLB and DAB fractions (Fig. 3C.3). Therefore, a change in the FLB fraction on treatment with penicillin has the potential to impact the interactions between the FLB and the DAB fractions. This may also be true for the other diatom cultures analyzed since their DAB isolates also showed a range of antibacterial activity, for e.g., the isolates from *Thalassiosira* sp. stimulated bacterial growth in these experiments (Fig. 3C.3, Table 3C.2). These results, coupled with the lower growth rates of near-axenic *A. coffeaeformis*, *Coscinodiscus* sp. and *Thalassiosira* sp. in the penicillin treatments (Fig. 3C.1a,c,e) suggest that inter-species interactions among bacteria play an important role in influencing diatom growth.