RESEARCH ARTICLE

The effect of antibiotics on diatom communities

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Effect of antibiotics (penicillin (P), streptomycin (S) and chloramphenicol (C)) on benthic diatom communities was evaluated using a modified extinction–dilution method. The high antibiotic combinations (2PSC and PSC) reduced diatoms by 99–100% and favoured emergence of yeast, probably due to high concentrations and synergistic effects. Changes in diatom communities in the individual antibiotic treatments were either direct (chloramphenicol and potentially streptomycin) or bacteria-mediated (penicillin). This study suggests that investigations on the fate of antibiotics in antibiotic-polluted and natural environments must consider effects across trophic levels and particularly, diatoms, the base of aquatic food webs.

Keywords: Antibiotics, antibiosis, bacteria, diatoms.

ANTIBIOTICS, substances of microbial origin, that target other microorganisms, have been widely studied. Since the first antibiotic, penicillin, was discovered accidentally in 1928 by Alexander Fleming, many other antibiotics have been discovered, chemically modified and synthesized *in toto* as well. Initially, antibiotics were thought to have a primary ecological role of inhibiting the growth of competitors[1]. Subsequently, the functioning of antibiotics as signalling molecules that modulate gene transcription at low concentrations was recognized[2]. Such signalling effects (antibiosis) may have beneficial consequences for bacteria that modulate the interactions within microbial communities[2,3]. Long *et al.*[4] have reported that antibiosis may significantly affect particle-associated bacterial communities and regulate the biochemical fate of particulate organic matter in oceanic environments. Such phenomena also extend across trophic levels[5]. This is especially interesting in the context of diatoms, the base of aquatic food webs, which are closely associated with bacteria at the genome, cellular and system levels[6–8].

The present study attempts to address how antibiotics affect diatom communities. We focused on microphytobenthic diatom communities, dominant primary producers in intertidal environments. Sediment samples were treated with various concentrations of antibiotics [penicillin (P), streptomycin (S) and chloramphenicol (C)], individually and in combination. Penicillin inhibits cell-wall peptidoglycan synthesis. It is effective mainly against Gram-positive bacteria. It affects the metabolism of Gram-negative rods as well, by inhibiting the activity of the penicillin-binding proteins[9]. Streptomycin and chloramphenicol inhibit protein synthesis, streptomycin by binding to the 30S ribosomal subunit and chloramphenicol by binding to the 50S ribosomal subunit[10]. Among these three antibiotics, penicillin cannot act on diatoms directly, whereas streptomycin and chloramphenicol have the potential to affect diatoms directly by inhibiting protein synthesis in diatom organelles (70S)[11]. Inhibition of photosystem II in diatoms by chloramphenicol is also known[12]. Thus, the different antibiotics used represented different types of antibiosis (qualitative effects), whereas the various concentrations reflected different levels of antibiosis or bacterial suppression (quantitative effects). We attempt to answer the following questions: (1) How do diatom communities respond to different types of antibiosis? (2) Are antibiotic-mediated changes in bacterial communities reflected in diatom communities?

Materials and methods

Sampling

Sampling was carried out in December 2006 on a sand flat at Dias Beach (15°27′N; 73°48′E) located near Dona Paula Bay, India. 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[13–15]. 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.

Experimental design

The extinction–dilution method (most-probable-number method, MPN)[16] was employed for analysing 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[17]. Artificial sea water (MBL; developed at the Massachu-

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setts Biological Laboratory)[18] enriched with f/2 level of nutrients[19], f/2(MBL), was used as the basal diluent, to which penicillin, streptomycin and chloramphenicol were added in varying proportions (3*X*, 2*X*, *X* and *X*/10) individually (P, S and C) and in combination (PSC). 3*X* was used only for streptomycin. *X* was 0.2 mg ml^{-1}, 0.1 mg ml^{-1} and 0.02 mg ml^{-1} for penicillin, streptomycin and chloramphenicol respectively. These antibiotic concentrations were chosen to mimic conditions in highly polluted environments, e.g. untreated sewage water and aquaculture pond sediments, where antibiotics can enter the sediments directly without undergoing any purification process[20]. Such environments maintain a high level of pollution irrespective of antibiotic degradation, due to the constant input of antibiotics[21]. Antibiotic concentration in sediments tends to be comparatively high due to adsorption processes[22], and is also highly locally concentrated[21].

Antibiotic stability

To test the extent of antibiotic degradation during the experiment, the stability of penicillin, streptomycin and chloramphenicol was analysed, individually and in combination, in the absence and presence of autoclaved sediment, collected from the study area (sandy, 83% sand). Antibiotic stability was assessed in f/2 medium prepared in artificial sea water (MBL) and aged sea water (ASW). Antibiotics were dissolved in the respective diluents and two sets were maintained. Autoclaved sediment from the study area was added to one set. After 7 days of incubation, aliquots were assayed against a test culture using the well diffusion method[23]. 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 represented by sterile distilled water. Assays were carried out in triplicates. The diameter of the inhibition zone (total diameter of the inhibition zone minus diameter of the well) was measured after 24 h incubation at room temperature, that ranged from 26°C to 28°C. Results are presented as zone of inhibition (percentage compared to day-0 values).

Analysis of microphytobenthic diatoms

To determine the effect of antibiotics on microphytobenthic diatom communities, the sediment (1 g) was suspended in the different diluents at a concentration of 0.1 g sediment ml^{-1}. This stock was diluted tenfold up to 10^{-5} 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, the diatoms were analysed 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 standard taxonomic keys, detailed in D'Costa and Anil[24]. Diatoms < 10 μm 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^{-1} wet sediment) was calculated by selecting a set of three consecutive dilutions and referring to a statistical table[16]. The relative diatom density (cm^{-3} wet sediment) was obtained by multiplying the MPN with the apparent specific gravity of the wet sediment[25], which was determined separately.

Analysis of bacteria

For quantification 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^{-1} wet sediment. Culturable bacteria were isolated by spread-plating appropriate aliquots of diluted sediment on Zobell marine agar (ZMA) 2216 containing the respective antibiotics. Morphologically distinct 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 Determinative Bacteriology*[26].

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 data in the other antibiotic treatments failed with respect to requirements for parametric analysis, and were analysed through non-parametric Kruskal–Wallis tests. All these analyses were performed using the STATISTICA 8 software.

Univariate measures of the diatom community – Shannon–Wiener's diversity index (*H*′), Margalef's species richness (*d*) and Pielou's evenness (*J*′) were analysed using PRIMER software (version 5).

Results

Antibiotic stability

In f/2(MBL), in the absence of sediment, antibiotic stability ranged from 77% (streptomycin) to 100% (antibiotic combination; Figure 1 *a*). In the presence of sterile sediment, antibiotic stability was lower, ranging from 61% (chloramphenicol) to 88% (antibiotic treatment;

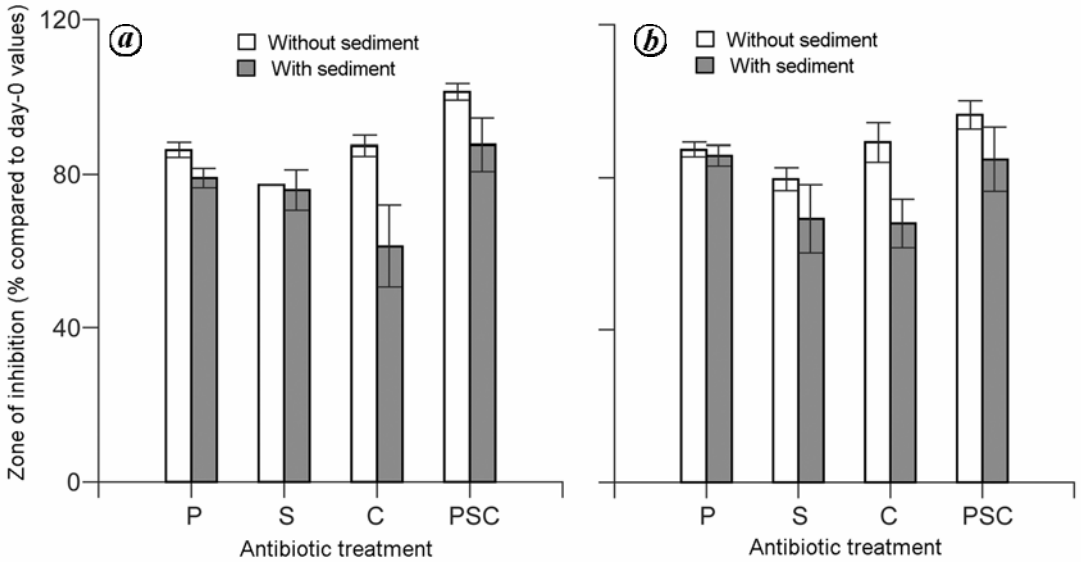

Figure 1. Antibiotic stability in the different treatments in (***a***) f/2(MBL) and (***b***) f/2(ASW), expressed in terms of zone of inhibition (percentage compared to day-0 values). P, 0.2 mg ml^{-1} penicillin; S, 0.1 mg ml^{-1} streptomycin; C, 0.02 mg ml^{-1} chloramphenicol; PSC, antibiotic combination treatment. Mean values ± SD are shown.

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

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

Figure 1 *a*). The trends obtained in f/2(MBL) were mirrored in antibiotic treatments in f/2(ASW; Figure 1 *b*).

Bacterial and diatom communities – control conditions

Bacterial abundance in control averaged 2.82×10^{10} bacteria g^{-1} wet sediment (data not shown). *Alcaligenes* and *Pseudomonas* (Gram-negative bacteria) and *Bacillus* (Gram-positive bacteria) were the dominant culturable bacteria (Table 1). A total of 27 diatom species belonging to 14 genera were recorded (Table 2), having an average diatom abundance of 4330 cells cm^{-3} wet sediment (data not shown). The dominant diatoms were *Amphora rostrata* and *A. coffeaeformis* (Figure 2).

Bacterial and diatom communities – antibiotic combination (PSC) treatments

Compared to control conditions, bacteria were suppressed to below detectable levels (≤ 1%) in the antibiotic combination treatments (Figure 3 *a*). No culturable bacteria were retrieved from the high (2PSC) and moderate (PSC) antibiotic combination treatments. Only yeast colonies were detected in these treatments (Table 1). Significant variation in diatom abundance was observed across the antibiotic combination treatments and control conditions (Kruskal–Wallis test, $P = 0.0172$). Diatom abundance varied from 100% reduction (no diatoms) in 2PSC to 82% in PSC/10 (532 diatoms cm^{-3} wet sediment; Figure 3 *b*). *A. rostrata*, *Nitzschia* sp. and UTDs dominated in PSC, whereas a wider range of diatoms were observed in

Table 2. Diatoms observed in control and different antibiotic treatments

Taxon	Treatment[a]													
	CONT	2PSC	PSC	PSC/10	2P	P	P/10	3S	2S	S	S/10	2C	C	C/10
Centrics														
Asteromphalus sp.							+							
Coscinodiscus sp.	+			+			+	+	+	+	+		+	+
Melosira nummuloides C.A. Agardh	+							+	+		+		+	
Odontella aurita (Lyngbye) C.A. Agardh	+			+	+		+	+	+	+	+		+	+
Odontella mobiliensis (Bailey) Grunow							+	+	+	+	+			
Odontella sp. 1	+						+	+	+	+	+	+	+	+
Odontella spp.	+							+	+	+	+	+	+	
Thalassiosira spp.	+			+			+	+	+	+	+			+
Pennates														
Achnanthes sp.	+							+			+	+	+	
Amphora coffeaeformis (Agardh) Kutzing	+			+			+	+	+	+	+	+	+	+
Amphora costata									+	+				
Amphora eunotia								+						
Amphora rostrata Wm. Smith	+		+	+			+	+	+	+	+			+
Amphora turgida Gregory	+			+			+	+	+	+	+	+	+	+
Amphora spp.	+			+			+	+	+	+	+		+	+
Cocconeis spp.	+			+			+	+	+	+	+		+	+
Cylindrotheca closterium (Ehrenberg) Lewin & Reimann				+										
Fragilaria sp.	+			+			+	+	+	+	+	+	+	+
Fragilariopsis spp.	+						+	+	+	+	+	+	+	+
Licmophora abbreviata	+													
Licmophora sp.											+			
Navicula crucicula (Wm. Smith) Donkin	+											+	+	
Navicula directa (W. Smith) Ralfs in Pritchard	+								+					
Navicula granii (Jorgensen) Gran	+								+					
Navicula subinflata Grunow	+			+			+	+	+	+	+			+
Navicula transitans var. *derasa* (Grunow, in Cleve and Grunow) Cleve	+			+			+	+	+	+	+	+	+	+
Navicula transitans var. *derasa* f. *delicatula* Heimdal	+								+	+	+			
Navicula vanhoffennii Gran	+													
Navicula spp.	+			+			+	+	+	+	+	+	+	+
Nitzschia panduriformis Gregory	+									+				
Nitzschia sp.	+		+	+		+	+	+	+	+	+	+	+	+
Synedropsis hyperborea (Grunow) Hasle, Medlin & Syvertsen	+							+	+	+	+		+	
Thalassionema frauenfeldii (Grunow) Hallegraeff									+					
Thalassionema nitzschioides (Grunow) Mereschkowsky	+			+			+	+	+	+	+		+	+
Unidentified diatoms	+		+	+		+	+	+	+	+	+	+	+	+

[a]Treatment details are given in the text.

PSC/10 (Figure 2). Species richness (*d*), Pielou's evenness (*J′*) and Shannon–Wiener diversity (*H′*) in the antibiotic combination treatments were lower than the control, except for evenness in PSC (Figure 4).

Bacterial and diatom communities – penicillin treatments

Bacteria were suppressed to 1–17% of control values in the penicillin treatments (Figure 3 *a*). The bacteria isolated from the 2P diluent lost viability on subculturing. Shifts in the culturable bacteria were observed in the other penicillin treatments (Table 1). Diatom abundance varied significantly compared to control conditions (Kruskal–Wallis test, $P = 0.0234$) and ranged from 0.1% to 38% of control values (Figure 3 *b*). The dominant diatoms differed from control and from each other (Figure 2). *Odontella aurita* was the only diatom observed in 2P. *Nitzschia* sp. and UTD were recorded in P, whereas *A. rostrata, Navicula* sp. and UTD were observed in the

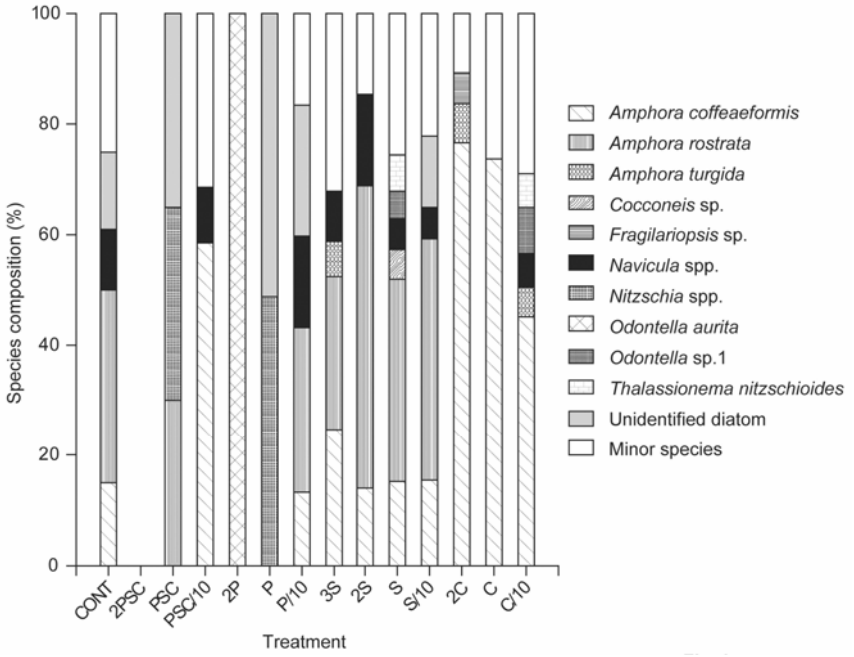

Figure 2. Species composition of diatom communities in the control and antibiotic treatments. Minor species represent the sum of the species contributing < 5%. CONT, Control; 2PSC, PSC and PSC/10, different concentrations of antibiotic combination treatment; 2P, P and P/10, 0.4 mg ml^{-1}, 0.2 mg ml^{-1} and 0.02 mg ml^{-1} penicillin; 3S, 2S, S and S/10, 0.3 mg ml^{-1}, 0.2 mg ml^{-1}, 0.1 mg ml^{-1} and 0.01 mg ml^{-1} streptomycin; 2C, C and C/10, 0.04 mg ml^{-1}, 0.02 mg ml^{-1} and 0.002 mg ml^{-1} chloramphenicol.

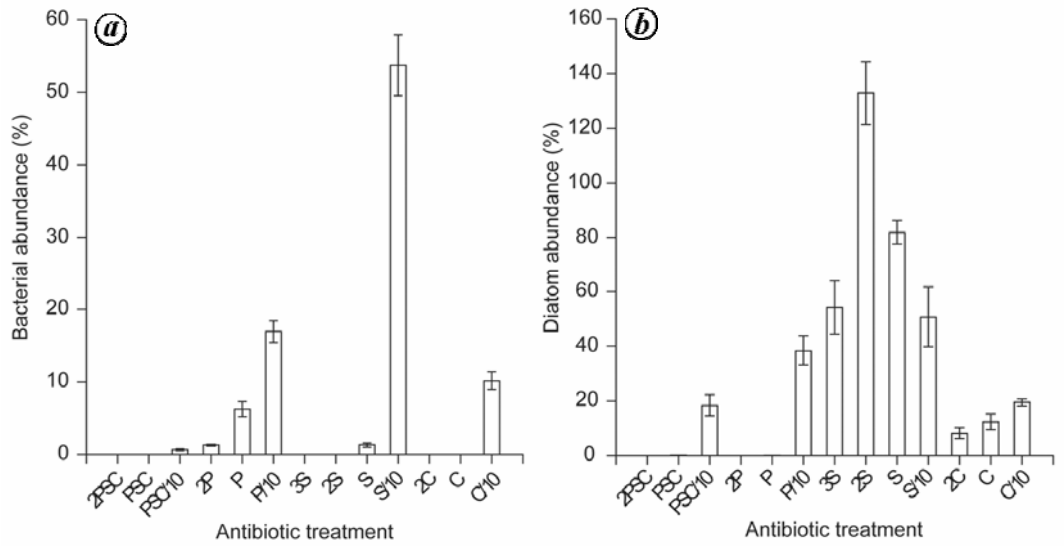

Figure 3. (***a***) Bacterial and (***b***) diatom abundance in the different antibiotic treatments, expressed as percentage of control values. Treatment abbreviations are the same as in Figure 2. Mean values ± SD are shown.

P/10 diluent (Figure 2). Species richness and diversity in all the penicillin treatments were low compared to control (Figure 4).

Bacterial and diatom communities – streptomycin treatments

Compared to control conditions, bacteria were suppressed to < 1–54% in streptomycin treatments (Figure 3 *a*). Shifts in the culturable bacteria were observed (Table 1). Diatom abundance varied significantly compared to control conditions (one-way ANOVA, $P = 0.0419$). The streptomycin treatments did not show a linear decrease in diatom abundance with increasing streptomycin concentration (Figure 3 *b*). The 2S treatment supported enhanced diatom emergence (5580 diatoms cm^{-3} wet sediment), approximately 133% compared to control (Figure 3 *b*). *A. coffeaeformis* and *A. rostrata* dominated in all the

streptomycin treatments (Figure 2). Species richness was lower than that under control conditions. Species evenness and diversity showed erratic trends (Figure 4).

Bacterial and diatom communities – chloramphenicol treatments

Bacteria were suppressed to < 1–12% in the chloramphenicol treatments (Figure 3 *a*). Shifts in the culturable bacteria were observed (Table 1). Significant variation in diatom abundance was observed across the chloramphenicol treatments and control conditions (Kruskal–Wallis test, $P = 0.0237$). The chloramphenicol treatments supported 8–19% diatom abundance (Figure 3 *b*). *A. coffeaeformis* was the most dominant diatom, irrespective of the concentration (Figure 2) and contributed 45–77% to the diatom community. Species richness, evenness and diversity in all the chloramphenicol treatments were low compared to control, except for evenness in C/10 (Figure 4).

Discussion

There are two aspects to be considered regarding antibiotic stability in the artificial sea water-based CONT. Firstly, the antibiotic stability profiles in f/2(MBL) were similar to those in f/2(ASW), indicating the similarity between the artificial sea water used and natural aged sea water. Secondly, antibiotic stability was lower in the presence of sediment than in its absence, probably due to adsorption processes[22]. However, the residual antibiotic activity was sufficient to effect characteristic changes in associated bacterial and microphytobenthic diatom communities. The nature of these changes depended on the mode of action and concentration of the antibiotics.

The antibiotic combination treatment, characterized by three different modes of action, was the most effective in suppressing bacteria. The bacteria were reduced below/near detectable levels in the 2PSC and PSC treatments, and approximately < 1% in the PSC/10 treatment. Such high bacterial suppression values could be attributed to the synergistic effect of the antibiotics. Penicillin that inhibits peptidoglycan synthesis in mainly Gram-positive bacteria, makes it easier for streptomycin and chloramphenicol to enter the cell[27]. An interesting trend observed was the emergence of yeasts in the high and moderate antibiotic combination treatments. Yeasts are important members of the microbial community in soil and have diverse degradative abilities[28]. Possibly, the elimination of bacteria to below detectable/or very low levels when treated with 2PSC and PSC favoured the development of these organisms, suggesting a shift in trophic dynamics. Examples of suppression of bacteria leading to emergence of other groups abound. For example, thraustochytrids were observed in the high concentration penicillin (2P) treatment, which reduced bacteria to 1% of control values. Also, when diatom cultures were treated with antibiotics to suppress bacterial growth, fungal growth in the cultures was found to increase (pers. obs.). The 'synergistic' effect of the antibiotic combination was also apparent in the 99–100% reduction in diatoms (Figure 3 *b*), changes in species composition, richness, evenness and diversity (Figures 2 and 4).

The antibiotics used have different effects on diatom communities. Chloramphenicol is a known inhibitor of diatoms. Streptomycin has the potential to affect diatoms directly through inhibition of protein synthesis in diatom organelles. On the contrary, penicillin can affect diatoms only through bacteria. Therefore, it is important to remember that there is another layer of complexity to consider in the case of diatom communities, i.e. diatoms may be affected directly by antibiotics as well as through changes in the bacterial component/community.

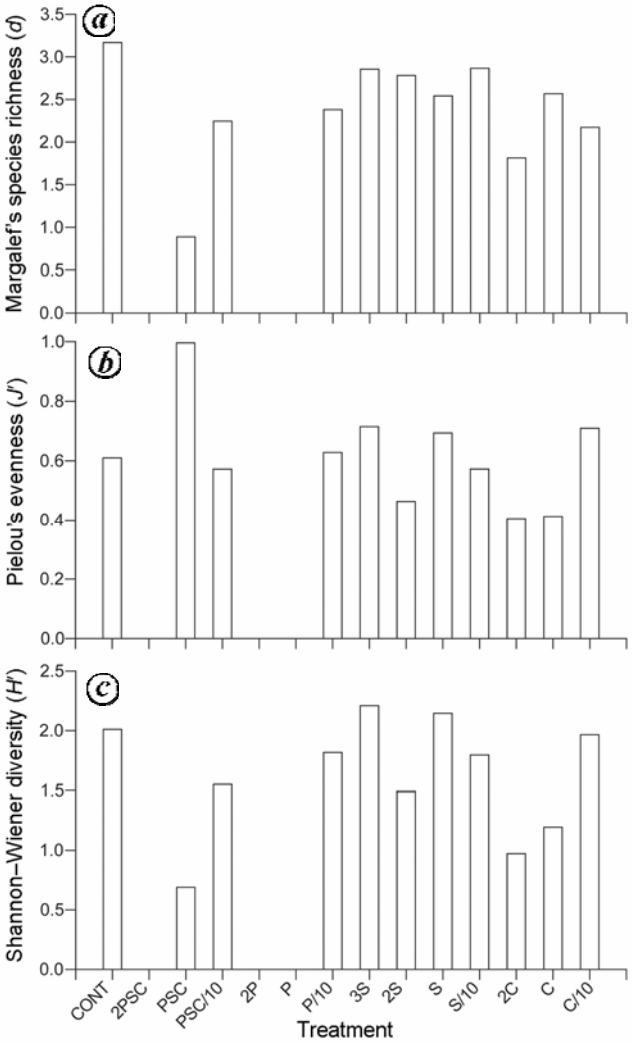

Figure 4. (***a***) Species richness, (***b***) Pielou's evenness and (***c***) Shannon–Wiener diversity of the diatom community in the different treatments. Treatment abbreviations are the same as in Figure 2. Mean values are shown.

Considering the bacterial community, suppression as well as shifts in the dominant culturable bacteria (*Alcaligenes*, *Alteromonas*, *Bacillus* and *Pseudomonas*) were noted in the different individual antibiotic treatments (Figure 3 *a*; Table 1). The dominant culturable bacteria identified in this study are known to lyse algae, either through direct contact or through production of dissolved algicidal compounds[29]. Therefore, changes in the dynamics of these bacteria may significantly contribute to changes in diatom communities when treated with antibiotics. In this context, it must be remembered that focusing on the total bacterial community in future studies will provide a more comprehensive understanding of antibiotic-mediated changes.

Such changes in the bacterial community are especially important in the penicillin treatments, which as mentioned before, can affect diatoms only through bacteria. Diatoms were reduced by 62–99.9% in the penicillin treatments. An interesting feature was the marked shifts in the species composition of the diatom community, mainly the emergence of only one diatom species (*O. aurita*) in the high penicillin concentration treatment (Figure 2; Table 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 (pers. obs.). Penicillin G inhibits mainly Gram-positive bacteria[10], which are surprisingly diverse in coastal ecosystems[26]. Gram-positive bacteria were originally thought to comprise a minor fraction (5%) of bacterial communities in the ocean[1]. However, research involving the use of low-nutrient media[30–32], 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[30,31] and are prolific producers of bioactive metabolites[33]. In view of our results, it is possible that this group of bacteria plays a significant role in modulating diatom communities and may provide a pathway through which penicillin affects diatom communities. However, the role of Gram-negative bacteria also needs to be studied, as penicillin can influence their metabolism by blocking penicillin-binding proteins[9]. Additionally, the mechanisms through which antibiotics affect diatoms through bacteria, need to be elucidated in future studies.

Compared to penicillin, streptomycin and chloramphenicol are relatively broad-spectrum antibiotics. They affect both Gram-positive and Gram-negative bacteria[10]. Chloramphenicol affects diatoms directly, whereas streptomycin has the potential to affect diatoms through inhibiting protein synthesis in diatom organelles. Diatom community structure differed in both these sets of treatment. In the chloramphenicol treatments, diatoms were reduced to 8–19% of control values (Figure 3 *b*). *A. coffeaeformis*, one of the dominant diatoms in the study area[15], appeared to be the most resistant to chloramphenicol. It dominated in all the chloramphenicol treatments, irrespective of the concentration (Figure 2). In the streptomycin treatments, diatoms did not show a dose-dependent response to different concentrations of streptomycin (Figure 3 *b*). The enhanced diatom growth in the 2S treatment (approximately 133% compared to control) suggests that the diatom community is not directly affected by streptomycin. Bacteria were also presumably not important because the enhanced diatom abundance was observed even though bacteria were suppressed to < 1% of control values and showed shifts in culturable bacteria. It is also possible that bacterial inhibition by streptomycin relaxed the constraints on diatom growth, and therefore resulted in higher diatom abundance compared to that in the control. That streptomycin did not affect diatom communities in a major manner is also obvious in the one-way ANOVA between the control and streptomycin treatments. This was contrary to the trend observed in the other antibiotic treatments.

From the above, it is clear that diatom communities respond differently to specific antibiotics. Another intriguing aspect to be considered is the differing response of the diatom communities to the various levels of antibiosis. This is clearly discernible in the occurrence profiles of *A. coffeaeformis* in the antibiotic combination and penicillin treatments. *A. coffeaeformis* was sensitive to the 2PSC, PSC, 2P and P treatments, and was recorded only in PSC/10 and P/10 (Table 2). This suggests the relevance of the antibiotic concentration used, mainly in the context of antibiotics having concentration-dependent roles. Antibiotics inhibit competitors at high concentrations and function as signalling molecules that influence metabolism at low concentrations[2,34].

The effect of penicillin, streptomycin and chloramphenicol on diatom communities must also be considered in the context of their diverse ecological roles. Penicillin and other β-lactam antibiotics are derived from fungi and actinomycetales, and sometimes from bacteria[33]. Aminoglycosides (the group to which streptomycin belongs) are produced exclusively by actinomycetales[33], whereas chloramphenicol is produced naturally by a bacterium, *Streptomyces venezuelae*. In this context, the results with penicillin, streptomycin and chloramphenicol also indicate the system-level interactions between diatom communities and specific microbial groups. The effects of these antibiotics on community-level responses of diatoms will give us new insights into the interactions between diatoms, fungi and actinomycetes in an ecosystem.

Conclusion

Diatom communities showed distinct changes in abundance and species composition when treated with different antibiotics. Direct effects on diatoms (chloramphenicol and potentially streptomycin) as well as bacteria-mediated

effects (penicillin) were observed, depending on the mode of action and the concentration of the antibiotics. In view of these results, we suggest that future studies on antibiotics in antibiotic-polluted as well as natural environments, must widen their scope to include not only bacteria but also diatoms, with which they are closely associated. The influence of antibiotics across other trophic levels also needs to be carefully monitored.

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