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Penicillin-mediated changes in viable, benthic diatom assemblages – insights about the relevance of bacteria across spatial and seasonal scales

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Abstract. Penicillin, a β -lactam antibiotic, cannot affect diatoms directly but does so through bacteria. Its effect on viable, benthic diatom assemblages in a tropical environment was evaluated across spatial (intertidal sandflat, mangrove site, port environment) and seasonal (post-monsoon, pre-monsoon, monsoon) scales. Penicillin treatment resulted in bacterial suppression and reduction of diatoms at the intertidal and mangrove sites having stable, pennate-dominated assemblages. Diatom response at the port site, dominated by transient, centric diatoms, ranged from total inhibition to enhancement. The monsoon diatom assemblages, differing in composition from that in other seasons, showed reduced susceptibility to penicillin. These observations highlight the bacterial influence on diatom assemblages and also the significance of species composition of diatom assemblages in these interactions. Epipsammic diatoms (*Cocconeis*, *Grammatophora* and *Thalassionema*) were more sensitive to penicillin compared to epipelagic forms probably due to (1) their inability to escape unfavourable bacterial metabolites because of limited diffusion in benthic environments, and (2) interference of penicillin with the bacterial ‘conditioning’ film required for their attachment. Overall, the observations from this preliminary, ecological study provide insights into the relevance of bacteria in influencing viable, benthic diatom assemblages across spatial and seasonal scales and emphasize the need for future mesocosm experiments on these aspects.

Additional keywords: bacteria, benthic diatoms, epipelagic, epipsammic, monsoon, penicillin.

Introduction

Microphytobenthic diatom assemblages serve as significant regulators of energy flow in intertidal foodwebs (Kuipers *et al.* 1981). They secrete a substantial proportion of fixed carbon in the form of exudates (Goto *et al.* 1999), which may function as a direct link between algae and bacteria. Rapid consumption of colloidal carbohydrates and glucan (photosynthetic storage products of diatoms) has been reported by Smith and Underwood (1998). Through an *in situ* ^{13}C pulse-chase experiment, carried out at 2 intertidal sites, Middelburg *et al.* (2000) have confirmed that this linkage is not only direct but also rapid, with carbon transfer from benthic algae to bacteria peaking after one day incubation.

Microphytobenthic diatom assemblages also contribute considerably to sediment stabilization, along with bacteria (Wigglesworth-Cooksey *et al.* 2001; McMinn *et al.* 2005; Gerbersdorf *et al.* 2009; Lubarsky *et al.* 2010). This sediment 'biostabilization' occurs through the exudation of Extracellular Polymeric Substances (EPS), a naturally adhesive organic material consisting of polysaccharides, proteins, nucleic acids, lipids and humic acids. Mixed diatom and bacterial assemblages provided greater stability than individual communities (Lubarsky *et al.* 2010 and references therein). Thus, due to their pivotal role in sediment stabilization, both these groups are referred to as 'ecosystem engineers' (Gerbersdorf *et al.* 2009).

Most studies on microphytobenthic diatoms focus on seasonal and spatial changes, vertical migratory rhythms, survival strategies and regulatory factors including the tidal cycle (physical factor), nutrient supply (chemical factor) and grazing and bioturbation (biological factors) (MacIntyre *et al.* 1996; Barranguet *et al.* 1998; Sundback *et al.* 2000; Mitbavkar and Anil 2002; 2004; 2006; Koh *et al.* 2007; Jordan *et al.* 2008; Scholz and Liebezeit 2012). Very few studies (Middelburg *et al.* 2000; Makk *et al.* 2003; Haynes *et al.* 2007; Bruckner *et al.* 2008 and 2011) explore the interactions between bacteria and benthic diatoms and still less is known about the effects of bacteria on entire benthic diatom assemblages.

In an earlier study (D'Costa and Anil 2011), we conducted experiments using an antibiotic, penicillin, that inhibits bacteria but not diatoms. This was done to test the hypothesis that changes in bacterial communities will result in changes in structure of diatom assemblages. The relevance of bacteria was apparent in reduced abundance, species richness and altered species composition of the viable, benthic diatom assemblages when treated with penicillin. The probable mechanisms responsible for these penicillin-mediated changes are numerous. Theoretically, penicillin-mediated changes in diatom assemblages are attributed to bacterial influence. Bacteria stimulate diatoms

through vitamins, siderophores, cytokinins and inhibit the same through direct contact and proteases (Maruyama *et al.* 1986; Amin *et al.* 2012 and references therein). Bacteria also influence diatoms through EPS carbohydrates, proteins and dissolved free amino acids (Lubarsky *et al.* 2010; Bruckner *et al.* 2011). They are competitors for diatoms in the context of space and resources and could also be parasitic or pathogenic to diatoms (Desbois *et al.* 2010 and references therein). Bacteria also confound allelopathic interactions between phytoplankton through degradation of toxins (Hulot and Huisman 2004). These interactions are influenced by turbulence and mixing intensity (Hulot and Huisman 2004), and can therefore vary spatially and seasonally.

The investigation detailed in D'Costa and Anil (2011) was based on a single sampling in a monsoon-influenced, tropical environment. Considering that viable, benthic diatom assemblages at the study site vary spatially and with seasons (Mitbavkar and Anil 2002; 2006), it is imperative to study penicillin-mediated changes in such assemblages across spatial and seasonal scales. Therefore, the same study was replicated across three study sites - an intertidal sandflat, a mangrove area and an anthropogenically-influenced port environment, during different seasons (post-monsoon, pre-monsoon and monsoon). The following questions were addressed: (1) Does the effect of penicillin (indicative of bacterial influence) on viable, benthic diatom assemblages vary across spatial scales? (2) Do these effects vary seasonally, i.e., across an annual seasonal cycle?

Materials and methods

Study sites and sampling strategy

Sediment and water samples were collected from three locations along the west coast of India: an intertidal sandflat (Dias Beach), a mangrove environment (Chorao), and a port environment (Mormugao port). The intertidal sandflat (Dias Beach) is surrounded by the Mandovi and Zuari estuaries (15°27' N; 73°48' E). It is about 200 m long, sheltered on both sides by rocky cliffs and has a semi-diurnal tidal cycle. The Chorao island is located in the backwaters of the Mandovi estuary (15°25'-15°30'N - 73°45'-73°59'E) and has extensive mangrove cover (comprising 15 species) towards the western low-lying tip. The Mormugao port is situated at the mouth of the river Zuari (15° 25' N; 73° 47' E), and accounts for about 33% of India's iron ore export. These 3 study sites represent different tidal heights: mid-intertidal (sandflat; sediment cores were collected at the mid-tide level), upper intertidal (mangrove) and sub-tidal (port).

The South West (SW) monsoon is the main source of climatic variation. A considerable amount of freshwater is added to the surrounding estuaries due to precipitation and run-off from terrestrial sources during the SW monsoon. Different seasons with characteristic environmental characteristics

can be discerned: pre-monsoon/summer (February-May), monsoon (June-September) and post-monsoon (October-January). These study sites were sampled in January 2005 (post-monsoon I), May 2005 (pre-monsoon/summer), September 2005 (monsoon) and January 2006 (post-monsoon II) to include an entire seasonal cycle and a repetition of one season.

Sediment and water sampling

At the intertidal sandflat and mangrove sites, surface sediment (0-5 cm) was collected in triplicate using PVC cores (inner diameter 2.6 cm). Separate sediment cores of the same dimensions were collected for analysis of specific gravity. At the port site, sediment samples were collected using a modified van Veen grab (0.04 m² grabbing area). The grab was equipped with flaps on the top side from which sediment cores could be collected. However, due to regular dredging at this site, the accumulated loose sediment was ~2 cm thick. Therefore, undisturbed cores could not be collected. Sediment was scooped out from the grab and stored in plastic packets wrapped in aluminium foil at 5°C. Chlorophyll *a* was analyzed spectrophotometrically using the 90% acetone extraction method (Parsons *et al.* 1984) and expressed as µg g⁻¹. Sediment texture (in terms of percentage of sand, silt and clay) was analyzed through pipette analysis (Buchanan 1984).

Surface water and interstitial water (at the intertidal sandflat) were sampled for the analysis of environmental variables. Interstitial water was sampled by digging the sediment with a shovel and allowing interstitial water to accumulate. Temperature, pH, dissolved oxygen (ml l⁻¹; Winklers method), salinity (psu; Mohr-Knudsen titration method) and Suspended Particulate Matter (SPM; mg l⁻¹; using 0.45 µm filter papers) were analyzed following Parsons *et al.* (1984) and Strickland and Parsons (1965). Dissolved inorganic nutrients (NO₃-N, NO₂-N, PO₄-P and Si; µM) were analyzed using spectrophotometric procedures detailed in Parsons *et al.* (1984). Chlorophyll *a* concentrations were estimated spectrophotometrically (as mentioned earlier) and expressed as µg l⁻¹.

Analysis of viable, benthic diatoms and bacteria

Microphytobenthic diatoms were analyzed through the extinction-dilution method (most-probable-number method, MPN) (Thronsen 1978), modified by the addition of penicillin. Artificial seawater (MBL) (Cavanaugh 1975) enriched with f/2 level of nutrients (Guillard and Ryther 1962) was used as the basal diluent and will henceforth be termed control. Penicillin was incorporated in the control diluent at 0.2 mg ml⁻¹.

In the original MPN method (Thronsen 1978), sediment samples are diluted with a seawater-based culture medium at an initial concentration of 0.1 g ml⁻¹. This suspension is diluted ten-fold up to 10⁻⁵. One ml of each dilution (i.e., 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵) is dispensed into 5 replicate wells

of a 24-well NUNCLON culture plate. The plates are incubated at 25 ± 1 °C with a 12:12 h light: dark cycle for 7 days to allow diatoms in the sediment to grow. Emergence of diatoms in these samples could be from two sources: (1) multiplication of vegetative cells and (2) germination of resting stages. Subsequently, the 24-well plates were checked microscopically for diatoms at 100X and 200X magnifications (and 400X when necessary) using an inverted microscope. Viability of diatoms was confirmed by observation of autofluorescence. Diatoms were identified based on Desikachary and Prema (1987), Desikachary *et al.* (1987a and b), Horner (2002), Subrahmanyam (1946) and Tomas (1997). The wells in which viable diatoms occurred were marked as positive. Scores for each species were measured in terms of number of positive wells; this number ranged from 0 to 5. All the wells (sets of 5 replicate wells for each dilution) were checked. For quantification of diatoms, three consecutive dilutions were selected (10^{-2} , 10^{-3} and 10^{-4} in this case). The number of positive wells in each dilution were combined in descending order, for e.g., 4-2-0 meaning 4, 2 and 0 positive wells in 10^{-2} , 10^{-3} and 10^{-4} respectively. These values were compared with a statistical table (Thronsdon 1978) to obtain the MPN of diatoms in the sediment sample (MPN g^{-1} wet sediment). The relative diatom density (cm^{-3} 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.

The extent of bacterial suppression was assessed by spread plating 0.1 ml aliquots of diluted sediment on Zobell Marine Agar (ZMA) 2216 with and without penicillin (in terms of CFU g^{-1}). Bacterial colonies were purified, maintained on ZMA 2216 slants and identified to genus level based on biochemical tests following Bergey's Manual of Systematic Bacteriology (Holt *et al.* 1994). All the analyses were carried out in triplicate.

Data analyses

The relationship between the environmental characteristics of the overlying water column and the viable, benthic diatom assemblages (in terms of no. of diatoms cm^{-3} wet sediment) was evaluated through Canonical Correspondence Analysis (CCA) (ter Braak 1995) using the Multi-Variate Statistical Package program version 3.1 (Kovach 1998). The viable, benthic diatom abundance data from the 3 replicates was averaged and used for CCA analysis. The data were \log_{10} -transformed. Rare species were down-weighted and the scores scaled by species. Through this analysis, the distinctness/similarities between the study sites could be assessed in addition to seasonal variations.

To provide additional information about changes in species occurrence, the number of species inhibited/enhanced was plotted. The univariate measures of the viable, benthic diatom assemblages

(Margalef's species richness, Pielou's evenness and Shannon-Wiener diversity) were analyzed separately for each replicate and averaged. The diatom abundance datasets were checked for significant variation ($p \leq 0.05$) across treatments and sampling periods using an *a priori* two-way crossed analysis of similarities (ANOSIM). This analysis produces the R statistic which is an absolute measure of the amount of separation between groups (Clarke and Gorley 2006). R values range from 0 [indicating complete overlap] to 1 [indicating complete segregation] (Clarke and Gorley 2006). Prior to ANOSIM, the data were standardized and square root-transformed. Square root transformation enables species having intermediate abundance to contribute to the similarity matrix, which will otherwise be dominated by the most abundant species (Clarke and Warwick 2001). The Bray-Curtis similarity coefficient was employed. The clustering of control and experimental treatments across the sampling periods was visualized in a Non-metric Multi Dimensional Scaling (NMDS) ordination plot, separately for each site, using the Bray-Curtis similarity matrix. These analyses were done using PRIMER (version 6) developed by PRIMER-E Limited, UK.

The bacterial abundance data under control conditions was assessed for significant variation across the sampling period using one-way ANOVA (normal data) and Kruskal-Wallis tests (non-normal data). This was done using STATISTICA 8 software. Since bacteria were reduced by >95% in the penicillin treatments, they were excluded from this analysis.

Results

Relationship between environmental variables and viable, benthic diatom assemblages

The intertidal and mangrove sites had a sandy and silty sediment texture respectively. Since the amount of sediment collected at the port site was meagre, we were unable to analyze it for sediment texture and relied on personal observations (rocky-sandy sediment texture). Therefore, sediment texture was not included in the CCA. The ranges of environmental variables observed at the study sites are provided (Table 1).

In the CCA biplots (Fig. 1), 4 axes were extracted that explained 82% of the relationship between the viable, control diatom assemblages and the environmental variables. Dissolved oxygen and salinity were the most important variables. Temperature, though included in the CCA analysis, is not depicted in the biplots because it is not one of the important environmental variables influencing the viable, benthic diatom assemblages. Its length is less than $1/10^{\text{th}}$ of either axis (one of the optional criteria for displaying environmental variables), and is therefore not displayed.

Distinct differences between the study sites were evident in the station biplot (Fig. 1a). The intertidal sandflat sampling periods clustered together. It had high dissolved oxygen, salinity and low phosphate and silicate concentrations. The port site had comparatively high nitrate and nitrite concentrations (Fig. 1a). The species biplot (Fig. 1b) reflected the trends observed in the station biplot. A group of species comprising *Biddulphia rhombus*, *Chaetoceros curvisetus*, *Chaetoceros subtilis*, *Leptocylindrus danicus*, *Odontella sinensis*, *Paralia sulcata*, *Pseudo-nitzschia lineola* and *Thalassionema bacillare* showed preference for elevated nitrate and nitrite concentrations, conditions typical of the port site. In fact, most of these species were recorded at only the port site (Fig. 1b, Table 2).

Effect of penicillin on viable, benthic diatom assemblages and bacteria

All the 3 sites supported characteristically different viable, control diatom assemblages. The abundance of the control diatom assemblages at the intertidal sandflat ranged from 178 to 3,620 diatoms cm^{-3} wet sediment (Fig. 2a). Twenty six diatom species belonging to 12 genera emerged (Table 2). *Navicula* sp. was the most abundant, followed by *Cocconeis scutellum*, *Fragilariopsis* sp., *Grammatophora marina*, *Navicula transitans* var. *derasa* and *Thalassiosira* sp. (Fig. 3). Species richness, evenness and diversity were highest in pre-monsoon (Table 3). The majority of species that contributed > 4% to the benthic diatom assemblage occurred throughout the sampling period (Fig. 3). *Grammatophora marina*, *Navicula transitans* var. *derasa* and *Thalassionema nitzschioides* were the only exceptions; they were conspicuously absent in monsoon (Fig. 3). Compared to control assemblages, the penicillin treatments were characterized by (1) reduced diatom abundance (Fig. 2a), (2) reduced species richness, evenness and diversity (Table 3), and (3) marked inhibition of *Cocconeis scutellum*, *Grammatophora marina* and *Thalassionema nitzschioides* across seasons (Fig. 3). Certain species-specific traits were also noted. A centric diatom, *Thalassiosira* sp., had higher abundance in the penicillin treatment compared to control (in pre-monsoon) (Fig. 3), resulting in a <1 pennate:centric diatom ratio (data not shown). *Amphora costata* and *Amphora* sp. had similar abundance in control and penicillin treatments, but only in monsoon (Fig. 3).

At the mangrove site, abundance of the control diatom assemblages ranged from 538 to 7,959 diatoms cm^{-3} wet sediment (Fig. 2b). Forty one species from 19 genera were recorded (Table 2). *Navicula* sp. was the most abundant followed by *Amphora coffeaeformis*, *Amphora* sp., *Navicula transitans* var. *derasa* and *Thalassiosira* sp. (Fig. 4). Species richness, evenness and diversity were highest in monsoon, pre-monsoon and post-monsoon II respectively (Table 3). Most of the species that contributed > 4% to the benthic diatom assemblage at the mangrove site were recorded throughout the sampling period (Fig. 4). Notable exceptions to this trend included *Amphora costata*,

Amphora sp. and *Pleurosigma* sp. In fact, *Pleurosigma* sp. had highest abundance in the monsoon period (Fig. 4). In the penicillin treatments, diatom abundance was reduced (Fig. 2b), to 5-32% of control values (data not shown). Species richness and diversity were reduced, with the exception of diversity in monsoon; evenness was consistently enhanced (Table 3). Most diatom species had lower abundance in the penicillin treatments throughout the sampling period (Fig. 4). *Nitzschia panduriformis* was totally inhibited by penicillin throughout the sampling period. *Amphora coffeaeformis*, *Pleurosigma* sp. and *Skeletonema costatum* did not follow this inhibition trend during the monsoon period. Additionally, *Thalassiosira* sp. had enhanced growth in the penicillin treatment compared to control but only in the post-monsoon II season (Fig. 4).

At the port site, abundance of the control diatom assemblages ranged from 5 - 293 diatoms cm⁻³ wet sediment (Fig. 2c). Twenty five species belonging to 15 genera were observed (Table 2). Substantial numbers of centric diatoms were recorded mainly *Thalassiosira* sp., *Skeletonema costatum* and *Chaetoceros subtilis* (Table 2; Fig. 5). The occurrence of species was not consistent in terms of being recorded throughout the sampling period (Fig. 5). The monsoon period supported the highest abundance of many species (Fig. 5). When treated with penicillin, diatom abundance ranged from total inhibition (post-monsoon I) to enhancement (monsoon) (Fig. 2c). *Skeletonema costatum* and *Thalassiosira nitzschioides* were totally inhibited except during monsoon (Fig. 5). Diatoms were not observed in the penicillin treatment in post-monsoon I. Additionally, a single diatom species was recorded in control and penicillin treatments in post-monsoon II. Species richness, evenness and diversity values could not be calculated for these treatments. The available values did not indicate a clear trend (Table 3).

The abundance of the control bacterial community ranged from 4.24 X 10⁶ to 2.86 X 10⁷ CFU g⁻¹ wet sediment at the intertidal sandflat, 9.23 X 10⁶ to 7.46 X 10⁷ CFU g⁻¹ wet sediment at the mangrove site and 1.08 X 10⁶ to 6.67 X 10⁷ CFU g⁻¹ wet sediment at the port site (Fig. 6). These variations across seasons were significant at all the sites (intertidal sandflat, one-way ANOVA, p = 0.004; mangrove site, Kruskal-Wallis test, p = 0.0307; port site, one-way ANOVA, p = 0.0113). Penicillin treatment reduced bacteria at all the sites (Fig. 6). Corresponding shifts in the culturable bacteria were also observed (Table 4).

Comparison of the effect of penicillin on the viable, benthic diatom assemblages across the study sites

The study sites were compared in terms of the number of diatom species inhibited/enhanced across the sampling period (Fig. 7). At the intertidal sandflat, the minimum values of inhibited/enhanced

species were observed in monsoon (Fig. 7a). At the mangrove site, inhibited diatom species were highest in post-monsoon I and lowest in monsoon (Fig. 7b). Diatom species were enhanced (in number) only during the monsoon period (Fig. 7b). At the port site, maximum number of species were inhibited during post-monsoon I whereas maximum number of species were enhanced in monsoon (Fig. 7c). Comparison of the plots of number of species inhibited/enhanced across the 3 study sites indicated that the highest numbers of inhibited/enhanced diatom species were observed at the mangrove site (Fig. 7).

ANOSIM of the viable, benthic diatom assemblages indicated significant variation across treatments at the intertidal sandflat with an R value of 0.509 (Fig. 8a). Significant variation across sampling periods was also detected, with R values ranging from 0.664 (mangrove site), 0.773 (intertidal sandflat) to 0.834 (port site) (Fig. 8a-c).

Discussion

The extinction-dilution method (Thronsen 1978) modified by the incorporation of penicillin was employed to assess viable, benthic diatom assemblages. This method relies on 2 basic assumptions: the cells are homogeneously distributed in the sample, and all viable cells will grow under the culture conditions provided (Thronsen 1978). Being a culture method, it has several advantages over chemical-based methods such as the oxidised sediment-permanent slide method (Sabbe 1993). (1) It allows the observation of diatom cells unaffected by chemicals and preservatives. (2) It is easier to detect epipsammic diatoms attached to sand grains and sediment particles (for e.g., *Cocconeis*, *Fragilariopsis*) as they multiply in the culture medium. (3) It provides an opportunity to observe both - vegetative as well as resting stages of diatoms. Additionally, solitary resting cells of diatoms can be confirmed as viable by observation of diatom autofluorescence. (4) It involves a 7 day incubation period to allow for germination of diatom resting cells and multiplication. Due to this, the effect of penicillin, an antibiotic that is effective mainly against actively growing cells, on diatoms, can be easily inferred. However, this method does not give the exact number of diatom cells but rather enumerates the relative abundance of different taxa. This is because it is based on the presence/absence of diatoms and involves a statistical table (Thronsen 1978) and probability theory as part of its calculations (Harris *et al.* 1998). Still, the extinction-dilution method is advantageous compared to methods like ultrasonification that tends to rupture fragile forms, the lens-tissue technique that focuses on epipelagic diatoms and the HPLC method that identifies benthic diatom assemblages only up to group-level.

In this study, penicillin, a β -lactam antibiotic, is incorporated in the extinction-dilution technique. Penicillin is effective against bacteria (prokaryotes). It inhibits cell wall (peptidoglycan) synthesis in Gram-positive bacteria and also influences the metabolism of Gram-negative rods by inhibiting the activity of penicillin-binding proteins (Semenitz 1983). Diatoms, unicellular organisms, are considered as 'simple' eukaryotes and have a cell wall that is different from that of bacteria. Thus, penicillin cannot directly affect diatoms. Based on this, it can be inferred that changes in viable, benthic diatom assemblages when treated with penicillin provide insights about the influence of bacteria.

Effects of penicillin on the viable, benthic diatom assemblages: insights about the relevance of bacteria

Overall, the viable, benthic diatom assemblages showed a range of effects in response to penicillin, from total inhibition to enhancement (Fig. 2), and reduction in species abundance, richness, evenness and diversity (with a few exceptions) (Figs. 3-5; Table 3), highlighting the role of bacteria as a regulatory factor. Yet, significant differences in the control and penicillin diatom assemblages were observed at only the intertidal sandflat (ANOSIM; Fig. 8a). At the mangrove and port sites, the high standard deviation among replicates possibly confounded this trend (Figs. 4-5). As discussed above, such changes in the viable, benthic diatom assemblages could be attributed to the effect of penicillin on bacteria in the sediment samples. The mechanisms by which such changes in bacterial communities translate to diatom assemblages are numerous, as discussed by Bruckner *et al.* (2011). Bacteria can influence diatoms via soluble factors (for e.g., amino acids, vitamins), thereby controlling their growth, EPS production (in terms of concentration and composition) and adhesion strength. In turn, diatoms influence bacterial communities mainly through released nutrients. Bacterial growth and DNA synthesis may also be stimulated (Bruckner *et al.* 2011 and Amin *et al.* 2012 and references therein).

This investigation on the effect of penicillin on viable, benthic diatom assemblages focused mainly on the diatom component from an ecological perspective. Bacterial analysis was limited to counts of culturable bacteria in the control and penicillin treatments and identification of bacterial isolates to genus level based on biochemical tests. This preliminary analysis of the bacterial component indicated suppression of bacteria (Fig. 6) by 71-99% (percentage data not shown). For e.g., *Vibrio* emerged selectively in the penicillin treatments on most occasions at the intertidal sandflat (Table 4). Probably, the inhibition of competing bacteria by penicillin provided favourable conditions for proliferation of *Vibrio* which, in the natural environment, is regulated by bacterium-bacterium antagonism (Long *et al.* 2005). Shifts in *Alcaligenes* and *Pseudomonas*, which have

stimulatory effects on diatoms (Fukami 1997), were also noted. However, since only a fraction of the bacterial community is recovered through culture techniques, the quantitative bacterial estimations based on viable counts and identification of bacteria through biochemical tests (Holt *et al.* 1994) in this study, provide only an indication of the changes occurring in the bacterial community. To obtain a clear picture of the changes in the total bacterial community, molecular tools are preferable. In a previous study on a similar aspect (D'Costa and Anil 2011), we used Denaturing Gradient Gel Electrophoresis (DGGE) to analyze the bacterial community at the intertidal sandflat. Cluster analysis of the bacterial DGGE band pattern based on Bray-Curtis similarity and group average method revealed that the penicillin and control treatments occurred in two different groups which showed similarity only at ~ 25%. This further supports our speculations about different bacterial communities in control and penicillin treatments and necessitates its evaluation in future studies. Yet, species obtained through culture-dependent approaches are not always detected using molecular methods (Schäfer *et al.* 2002). In view of this, we suggest that future studies should use a combination of culture studies and molecular approaches.

Influence of bacteria on the viable, benthic diatom assemblages: spatial variations

The diatom assemblages showed site-specific responses to penicillin treatment, ranging from consistent reduction in diatom abundance at the intertidal and mangrove sites to an irregular pattern (inhibition and enhancement) at the port site (Fig. 2). In fact, two-way crossed ANOSIM revealed that the control and penicillin diatom assemblages were significantly different at only the intertidal sandflat (Fig. 8a). These spatial variations were also apparent in the specific diatoms consistently inhibited by penicillin at the study sites - *Cocconeis scutellum*, *Grammatophora marina* and *Thalassionema nitzschioides* (intertidal sandflat), *Nitzschia panduriformis* (mangrove site) and *Skeletonema costatum* and *T. nitzschioides* (port site) (Figs. 3-5). Species evenness of the viable, benthic diatom assemblages also reflected these spatial differences; it was reduced at the intertidal sandflat and enhanced at the mangrove site (Table 3). Since these values were only partially available at the port site, clear trends were not discernible.

The study sites supported distinct bacterial and viable, benthic diatom assemblages. The bacterial abundance at the study sites depended on sediment texture. Basically, bacterial abundance shows an inverse relationship with grain size (Dale 1974). Intertidal sediments, with a coarse sediment texture, have lower bacterial abundance compared to silty sediments (Fig. 6). The variation in sediment texture, in addition to environmental variables, will influence the bacterial communities in sediment (for e.g., restriction of *Vibrio* at the intertidal sandflat). These differences were probably

reflected in the different levels of bacterial suppression (Fig. 6) and influenced the response of viable, benthic diatom assemblages to penicillin.

Considering diatoms, pennate diatoms dominated at the intertidal and mangrove sites whereas transient, centric diatoms dominated at the port site. These variations reflected the contrasting environmental characteristics in the study sites. The intertidal sandflat had a sandy sediment texture, as mentioned earlier, and lower nutrient concentrations (Table 1). The mangrove site had a silt-rich sediment texture (Table 1) which, due to its small size fraction (2-63 μm) and high surface area, was favourable for diatoms. At both these sites – mid-intertidal sandflat and upper intertidal mangrove, sediments were not continuously submerged and were thus subjected to desiccation/loss of water. The effect of desiccation was pronounced for the intertidal sandflat due to its coarse, sandy sediment texture. Hydrodynamic forces are also stronger in the intertidal sandflat. Compared to the mangrove site, the anthropogenically-influenced port site had higher dissolved oxygen, salinity, pH, suspended particulate matter (Table 1) and a rocky-sandy texture. This site is sub-tidal, i.e., the sediment is continuously submerged under water. Thus, the benthic diatom assemblages may be light-limited. Additionally, the absence of an undisturbed substratum could contribute to the prevalence of centric diatoms and the wide range of responses of the diatom assemblages to penicillin, a pattern different from that observed in the species-stable intertidal and mangrove sites. This further highlights the influence of species composition of the diatom assemblages on their response to penicillin.

Influence of bacteria on the viable, benthic diatom assemblages: seasonal variations

Significant seasonal variation was observed at all the study sites (ANOSIM; Fig. 8). The R values of 0.834 (port site) and 0.773 (intertidal sandflat) indicated good segregation based on sampling period, also apparent in the ANOSIM NMDS (Fig. 8). The effect of penicillin on microphytobenthic diatom assemblages also varied across the three sites (Figs. 3-5). At the intertidal sandflat, the most striking change in structure of diatom assemblages in the penicillin treatments was observed in the pre-monsoon/summer months. Most pennate diatoms were inhibited whereas *Thalassiosira* sp., a centric, tythropelagic diatom was abundant (Fig. 3). In pre-monsoon, the viable, benthic diatom assemblage had the highest species evenness and diversity (Table 3). In diverse assemblages, positive interactions between species tend to be stronger, especially when evenness is high, i.e., many individuals contribute significantly to the community (Hillebrand and Cardinale 2004 and references therein). Such interactions between constituent diatoms, additionally influenced by bacteria through allelopathy, were probably responsible for the enhanced abundance of *Thalassiosira* sp.

The viable, benthic diatom assemblages during monsoon differed in composition from that in the other seasons. At the intertidal sandflat, *Grammatophora marina*, *Navicula transitans* var. *derasa* and *Thalassionema nitzschioides* were conspicuously absent in monsoon (Fig. 3). Multiple species had highest abundance during monsoon at the port site (Fig. 5). Similarly, *Pleurosigma* sp. had highest abundance during monsoon at the mangrove site (Fig. 4). The dynamic, transient nature of the viable, benthic diatom assemblages in monsoon was also apparent in the high standard deviation associated with species richness, evenness and diversity measures, especially at the intertidal sandflat (Table 3a). These differences in the viable, benthic diatom assemblages were reflected in the lower inhibition levels during the monsoon period at all the study sites (Figs. 3-5). In fact, the monsoon period also exhibited the highest number of enhanced species at the mangrove and port sites (Fig. 7). These monsoon-specific assemblages are most likely to have individuals that are able to survive the turbulence and fluctuations in physico-chemical variables associated with the monsoon and translate to reduced levels of susceptibility to penicillin and, in other words, bacteria.

Both post-monsoon periods exhibited pronounced differences in the number of inhibited species at the mangrove and port sites and the number of enhanced species at the intertidal sandflat (Fig. 7). Additionally, *Thalassiosira* sp. had higher abundance in the penicillin treatment compared to control conditions at the mangrove site, but only in post-monsoon II (Fig. 4). These differences could be attributed to the differing control diatom assemblages in both post-monsoon periods (Figs. 3-5) which, in turn, are influenced by the preceding SW monsoon. The SW monsoon is characterized by an increase in turbulence, water mixing, nutrient loads as well as a drop in salinity. In the context of the critical-rate hypothesis (Scheffer *et al.* 2008), rainfall frequency is also an important factor. The significance of the SW monsoon has also been recognised in similar observations for diatom and dinoflagellate communities in tropical environments (D'Costa *et al.* 2008; D'Costa and Anil 2010). Thus, the differences observed in the control and penicillin treatments in monsoon and across both post-monsoon periods can be understood in the context of the preceding monsoon and reflects the pivotal role of the SW monsoon in influencing these interactions in tropical environments.

Influence of bacteria on the viable, benthic diatom assemblages: epipsammic v/s epipellic diatoms

Species belonging to certain diatom genera (*Coscinodiscus*, *Cocconeis*, *Grammatophora* and *Thalassionema*) were sensitive to penicillin on most occasions. *Coscinodiscus* is a centric diatom, k-selected and slow-growing. Diatoms belonging to *Cocconeis*, *Grammatophora* and *Thalassionema* constitute the epipsammic diatoms at the intertidal sandflat. Epipsammic diatoms are usually attached to sand grains and are either immobile or capable of limited movement (Round 1965 and 1979). This proves to be a disadvantage in terms of interactions with bacteria since bacterial

metabolites tend to concentrate near their point of origin in benthic environments due to limited diffusion compared to the pelagic system. Contrary to this trend, epipellic diatoms (for e.g., *Navicula* and *Amphora* sp.) can move actively in the surface layers of sediment (Round 1965 and 1979), and have a higher chance of escaping unfavourable conditions/gradients. This could explain their comparatively lower sensitivity to penicillin. Additionally, penicillin may interfere with the bacterial ‘conditioning’ film required for attachment of epipsammic diatoms. This seems to be true for *Cocconeis*, a widely reported epiphyte (Buric *et al.* 2004). It grows tightly appressed to the substratum and is described as a ‘tightly attached alga’ (Cattaneo 1990). Even in the present study, *Cocconeis scutellum* was observed adhered to sediment grains at the intertidal sandflat. Bacteria may perform an important role in mediating its attachment to sediment grains. However, the distinction between epipsammic and epipellic diatoms is not absolute; some epipsammic diatoms are capable of slow movement. Also, certain genera (*Amphora*, *Navicula* and *Nitzschia*) have representatives in both groups (Scholz and Liebezeit 2012 and references therein).

Summarizing, penicillin-mediated changes in viable, benthic diatom assemblages, indicative of the influence of bacteria, showed clear spatial variations, mainly in terms of the species-stable intertidal and mangrove sites *versus* the anthropogenically-influenced port site, characterized by dominance of transient, centric diatoms. Seasonal variations were also apparent, mainly in the pre-monsoon and monsoon periods. Interestingly, epipsammic diatoms (*Cocconeis*, *Grammatophora* and *Thalassionema* species) were more sensitive to penicillin compared to epipellic forms. However, since the experimental approach used is novel, certain aspects need to be considered carefully. Firstly, direct effects of penicillin on diatom assemblages cannot be completely ruled out, without resorting to experimental studies with axenic diatom monocultures. Secondly, it must be noted that future studies must compare the conditions between the semi-cultivation extinction-dilution method and *in situ* natural environments, before extrapolating these results to natural assemblages. Yet, even with these limitations, this preliminary study serves as a baseline for future mesocosm experiments elucidating the mechanisms underlying diatom-bacterial interactions in benthic environments.

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Legends to figures

Fig. 1. Ordination diagrams for (a) sampling periods and (b) species based on Canonical Correspondence Analysis (CCA) of the viable, benthic diatom assemblages. The environmental variables (salinity, dissolved oxygen, nitrate, nitrite, phosphate and silicate) are indicated by arrows, labeled Sal, DO, NO₃, NO₂, PO₄ and Si respectively. PM-I, PreM, Mon and PM-II represent the post-monsoon I, pre-monsoon, monsoon and post-monsoon II sampling periods respectively. Species codes are presented in Table 2. These ordination diagrams depict the relationship between the environmental characteristics of the overlying water column and the viable, benthic diatom assemblages at the study sites. This analysis also allows us to examine the distinctness of the study sites (indicated by circled groups) and the seasonal variations in assemblages.

Fig. 2. Seasonal variation in abundance of the viable, benthic diatom assemblages in the control and penicillin treatments at the (a) intertidal sandflat, (b) mangrove site and (c) port site. Viable, benthic diatom abundance was analyzed using the extinction-dilution method (Thronsen 1978) and expressed as diatoms cm⁻³ wet sediment. Mean values + SD are shown. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II.

Fig. 3. Seasonal variation in the abundance of individual diatom species (diatoms cm⁻³ wet sediment) in the control and penicillin treatments at the intertidal sandflat. Mean values + SD are shown. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II. Species that contributed >4% are depicted.

Fig. 4. Seasonal variation in the abundance of individual diatom species (diatoms cm⁻³ wet sediment) in the control and penicillin treatments at the mangrove site. Mean values + SD are shown. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II. Species that contributed >4% are depicted.

Fig. 5. Seasonal variation in the abundance of individual diatom species (diatoms cm⁻³ wet sediment) in the control and penicillin treatments at the port site. Mean values + SD are shown. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II. Species that contributed >4% are depicted.

Fig. 6. Seasonal variation in bacterial abundance (CFU g⁻¹ wet sediment) in the control and penicillin treatments at the (a) intertidal sandflat, (b) mangrove site and (c) port site. Mean values + SD are shown. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II.

Fig. 7. Seasonal variation in the number of diatom species inhibited/enhanced in the penicillin treatment at the (a) intertidal sandflat, (b) mangrove site and (c) port site. PM-I: post-monsoon I; PreM: pre-monsoon; Mon: monsoon; PM-II: post-monsoon II.

Fig. 8. Non-metric Multi Dimensional Scaling (NMDS) plots based on ANOSIM diatom abundance data from the (a) intertidal sandflat, (b) mangrove site and (c) port site. In S1.1, the first and second digits represent the sampling period and replicate no. respectively. Overall, there are 4 sampling periods, denoted as S1 (post-monsoon I), S2 (pre-monsoon), S3 (monsoon) and S4 (post-monsoon II), and 3 replicates (1-3). The R and P (%) values for the ANOSIM analysis across treatments and sampling periods are also provided.

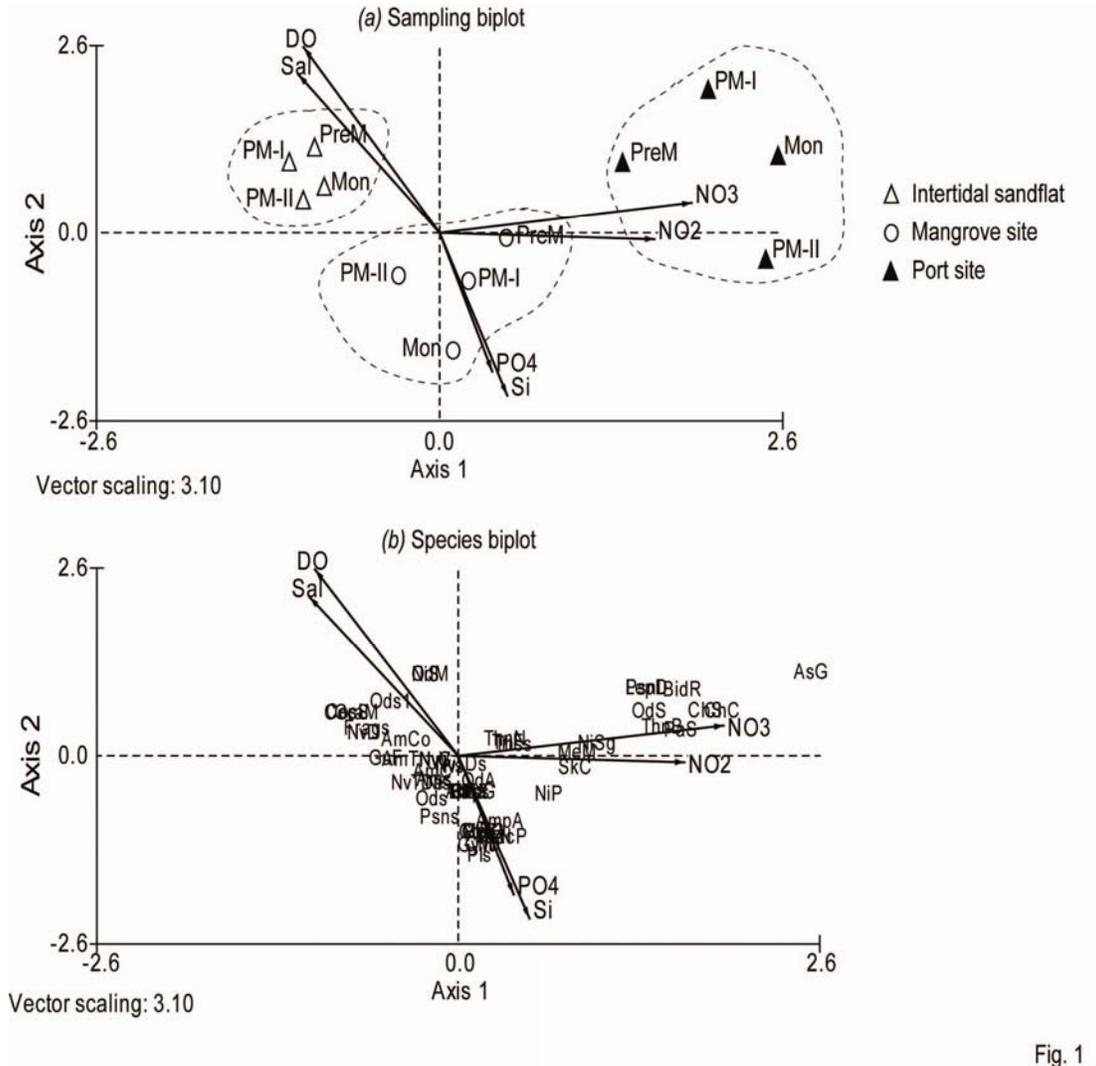


Fig. 1

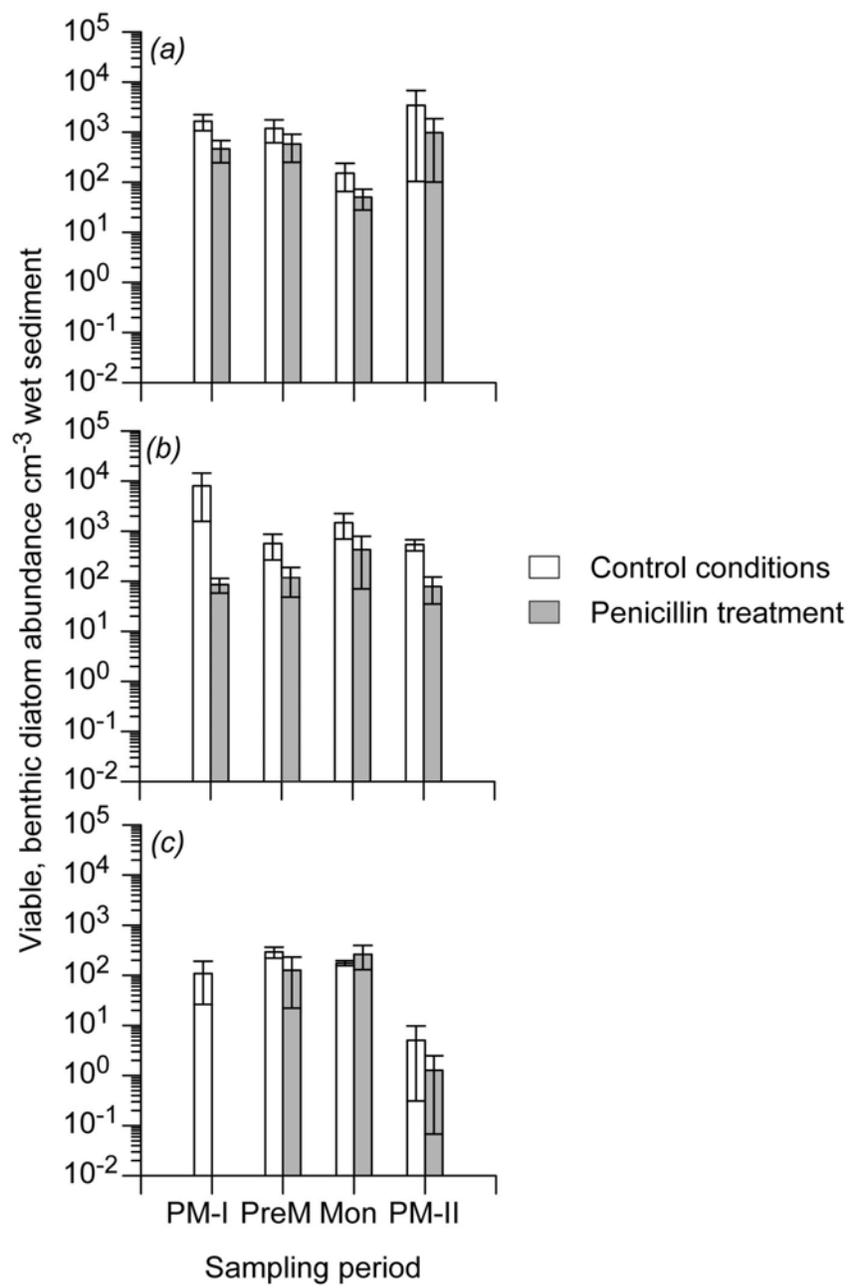


Fig. 2

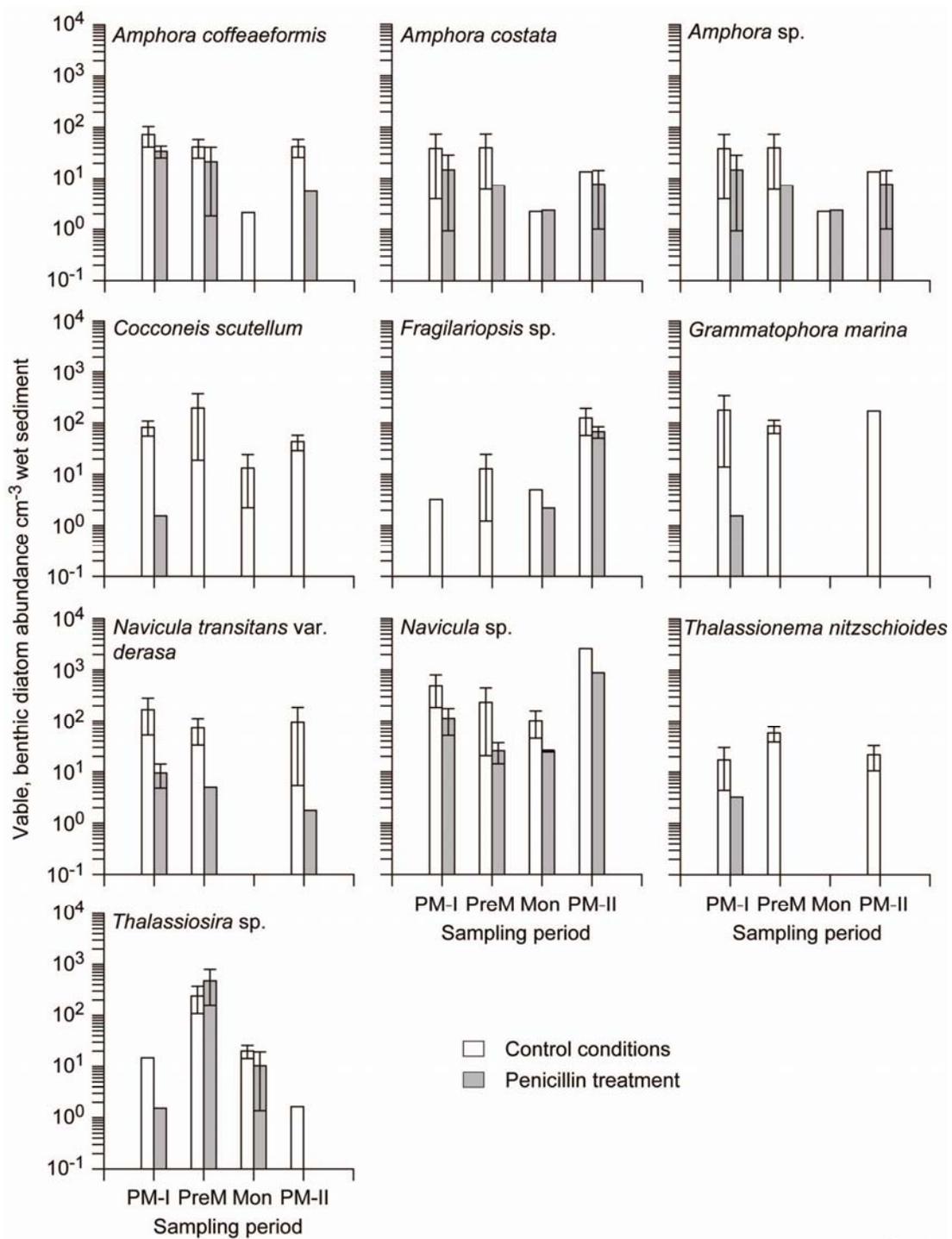


Fig. 3

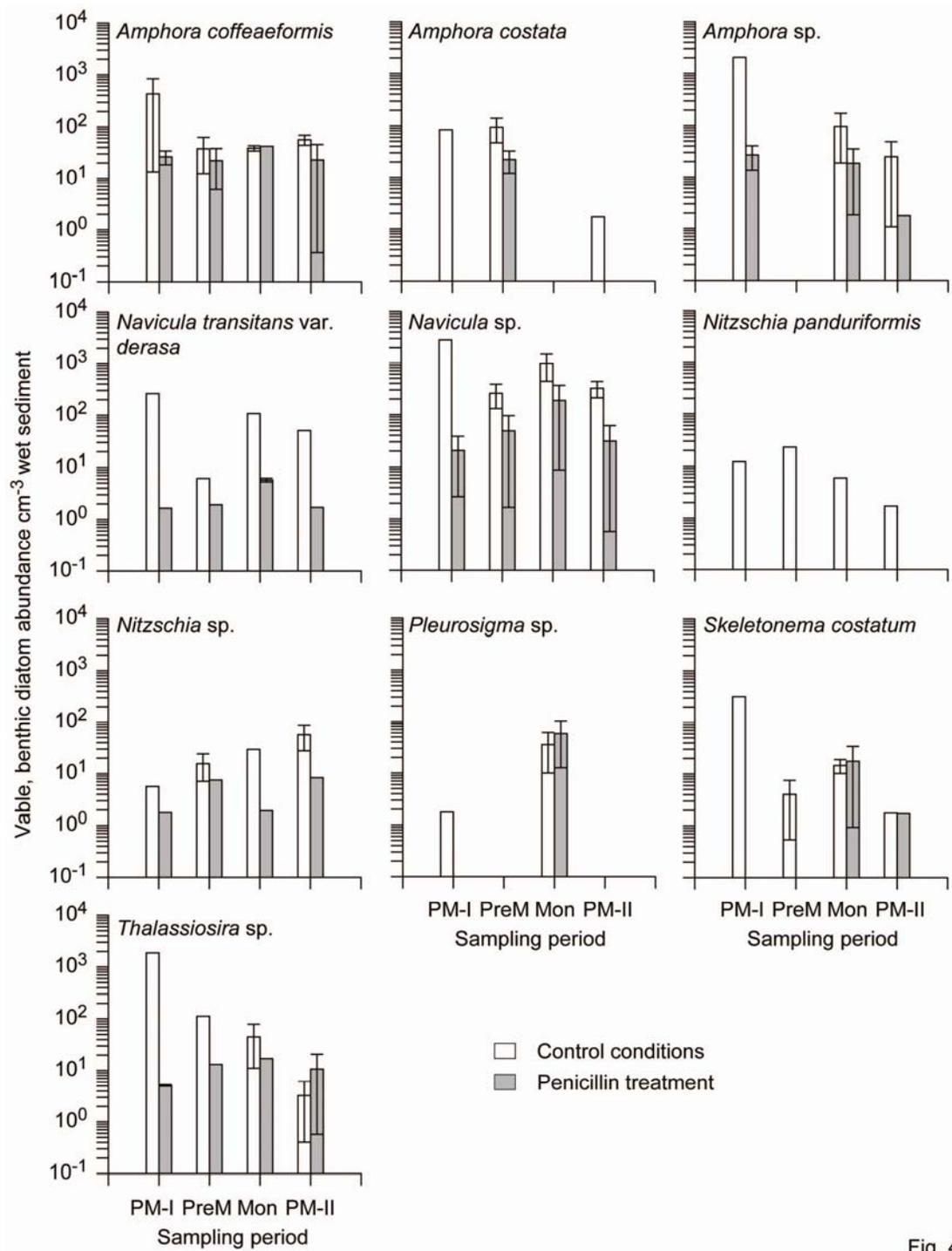


Fig. 4

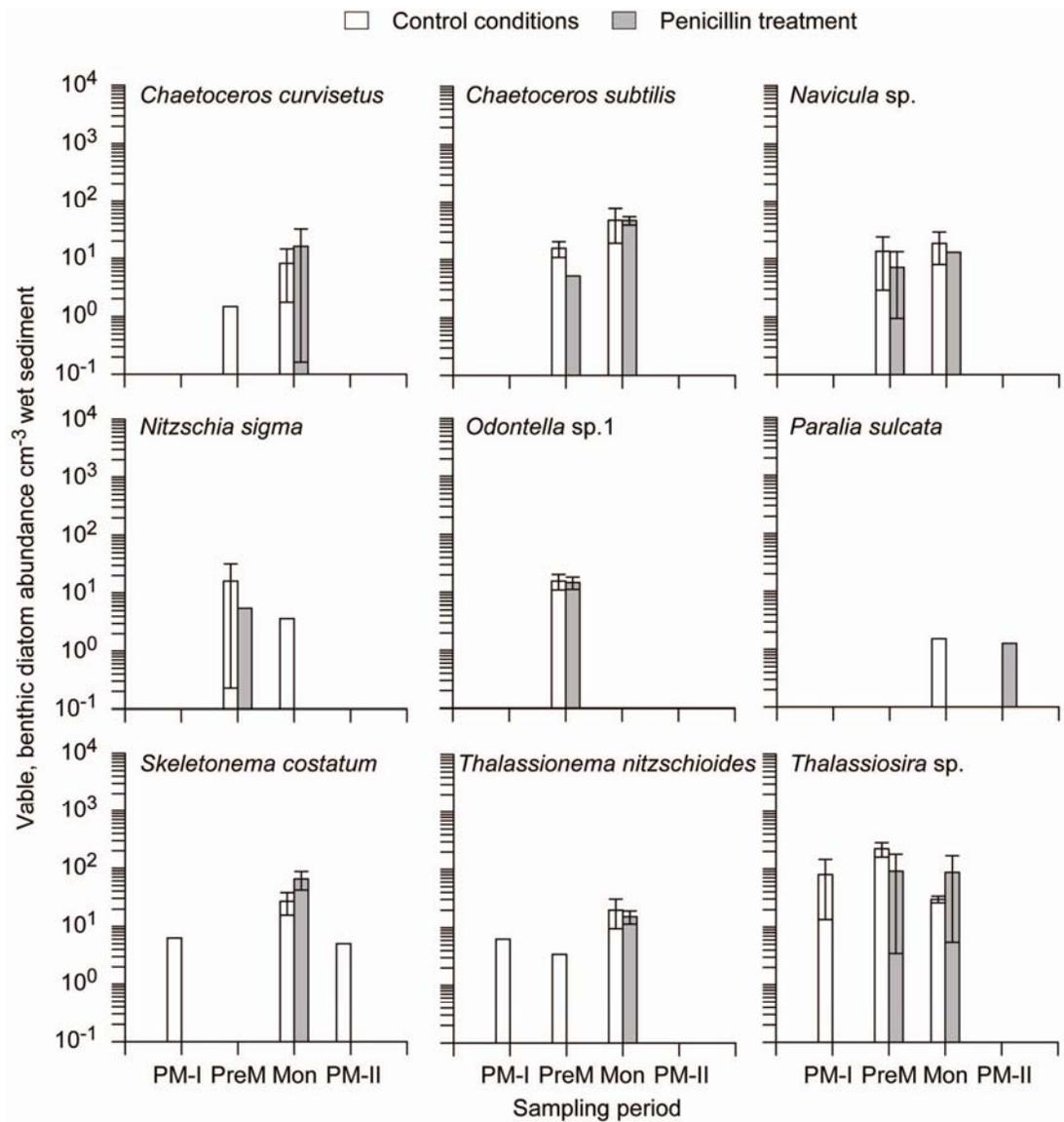


Fig. 5

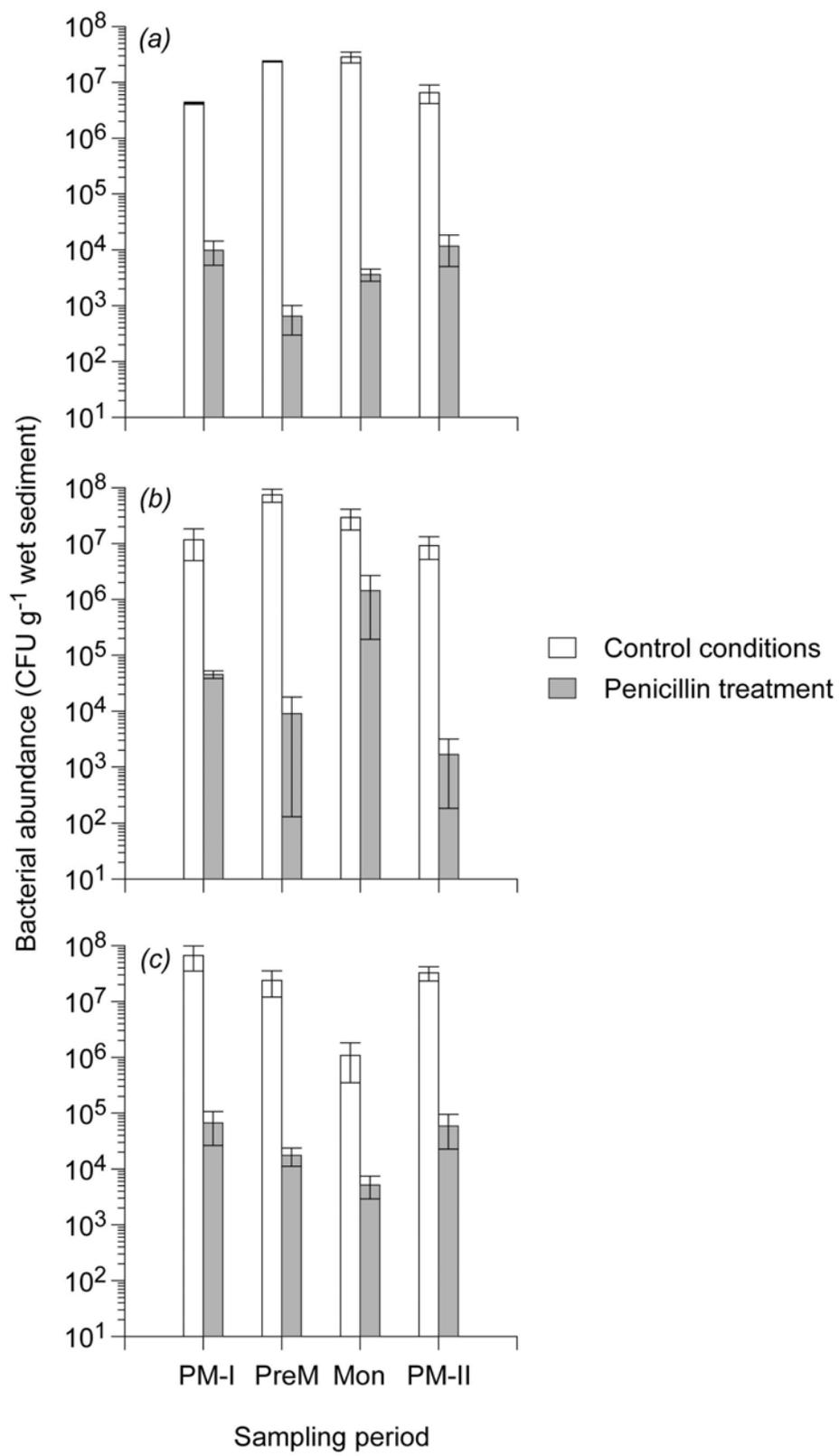


Fig. 6

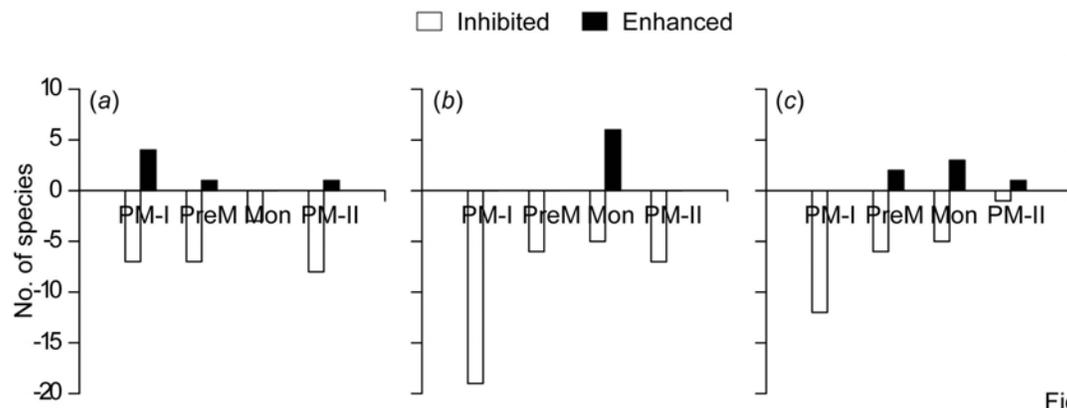


Fig. 7

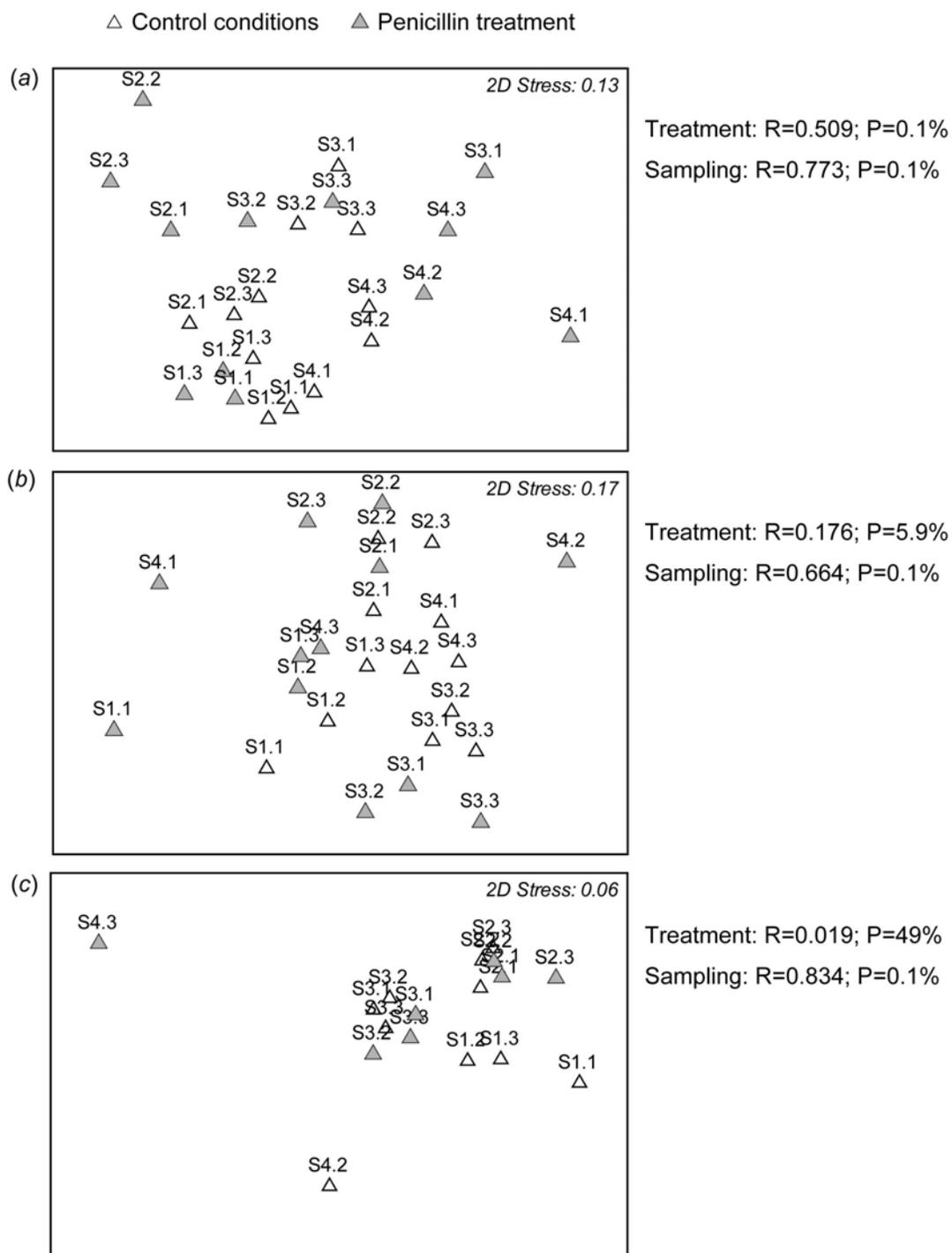


Fig. 8

Table 1. Range of environmental parameters in surface waters, interstitial waters and sediment at the three study areas

Parameter		Units	Intertidal sandflat	Mangrove site	Port site
Chlorophyll <i>a</i>	SW	$\mu\text{g l}^{-1}$	3-25	2-10	2-12
	IW	$\mu\text{g l}^{-1}$	3-9	-	-
	Sed	$\mu\text{g g}^{-1}$	0.3-3	8-15	5-10
SPM	SW	mg l^{-1}	17-36*	2-11*	8-39
	IW	mg l^{-1}	44-65*	-	-
Temp	SW	$^{\circ}\text{C}$	27-31	27-33	27-32
	IW	$^{\circ}\text{C}$	28-30	-	-
DO	SW	ml l^{-1}	2-4	2.2-2.8	3-4
	IW	ml l^{-1}	0.4-1	-	-
Salinity	SW	psu	31-36	8-32	18-36
	IW	psu	27-36	-	-
pH	SW	-	8.0-8.3	7.5-7.8	7.8-8.6
	IW	-	7.7-8.2	-	-
Nitrate	SW	μM	0.02-9	0.08-4	0.2-10
	IW	μM	0.2-1	-	-
Nitrite	SW	μM	0-0.3	0.03-0.3	0.2-0.4
	IW	μM	0.1-2	-	-
Phosphate	SW	μM	0.2-2	0.3-2	0.3-1
	IW	μM	0.2-3	-	-
Silicate	SW	μM	2-40	9-93	1-20
	IW	μM	8-43	-	-
Texture	Sed (sand)	%	55-838	19-28	-
	Sed (silt)	%	17-45	71-80	-
	Sed (clay)	%	0.04-0.1	0.1-0.4	-

SW, surface water; IW, interstitial water; Sed, sediment; * not analyzed during pre-monsoon.

Table 2. Viable, benthic diatoms observed in the control and penicillin treatments at the study sites during the four sampling periods

Taxon	CCA species code	Intertidal sandflat		Mangrove site		Port site	
		CONT	P	CONT	P	CONT	P
Centric diatoms							
<i>Biddulphia rhombus</i> (Ehrenberg) W. Smith	BidR		+	+	+	+	
<i>Chaetoceros curvisetus</i> Ehrenberg	ChC					+	+
<i>Chaetoceros didymus</i> Ehrenberg	ChD			+			
<i>Chaetoceros subtilis</i> Cleve	ChS					+	+
<i>Coscinodiscus lineatus</i> Ehrenberg	CosL	+	+				
<i>Coscinodiscus radiatus</i> Ehrenberg	CosR			+			
<i>Ditylum sol</i> Grunow in van Heurck	DitS						+
<i>Guinardia flaccida</i> (Castracane) H. Peragallo	GuF	+		+			
<i>Helicotheca tamesis</i> (Shrubsole) Ricard	HeT						+
<i>Leptocylindrus danicus</i> Cleve	LepD					+	
<i>Melosira moniliformis</i> C. Agardh	MeM			+	+	+	
<i>Melosira nummuloides</i> C. Agardh	MeN		+	+	+	+	
<i>Melosira</i> (C. Agardh) sp.	Mes			+			
<i>Odontella aurita</i> (Lyngbye) C.A. Agardh	OdA	+		+		+	
<i>Odontella mobiliensis</i> (Bailey) Grunow	OdM	+				+	+
<i>Odontella sinensis</i> (Greville) Grunow	OdS			+		+	
<i>Odontella</i> (C. Agardh) sp.1	Ods1	+	+			+	+
<i>Odontella</i> (C. Agardh) sp.	Ods	+	+	+	+		
<i>Paralia sulcata</i> (Ehrenberg) Cleve	PaS			+	+	+	+
<i>Skeletonema costatum</i> (Greville) Cleve	SkC			+	+	+	+
<i>Thalassiosira</i> (Cleve) sp.	Thss	+	+	+	+	+	+
<i>Triceratium favus</i> Ehrenberg	TriF					+	
Pennate diatoms							
<i>Amphiprora angustata</i> Hendey	AmpA			+	+		
<i>Amphiprora gigantea</i> Grunow	AmpG			+			
* <i>Amphora coffeaeformis</i> (Agardh) Kutzing	AmC	+	+	+	+		+
* <i>Amphora costata</i> W. Smith	AmCo	+	+	+	+		
* <i>Amphora turgida</i> Gregory	AmT	+	+	+			+
* <i>Amphora</i> (Ehrenberg ex. Kutzing) sp.	Ams	+	+	+	+	+	
<i>Asterionellopsis glacialis</i> (Castracane) Round	AsG					+	+
<i>Bacillaria paxillifera</i> (O. F. Muller) T. Marsson	BacP			+	+	+	
<i>Bacillaria</i> (J. F. Gmelin) sp.	Bacs			+			
<i>Climacosphenia</i> (Ehrenberg) sp.	Clis			+			
<i>Cocconeis scutellum</i> Ehrenberg	CocS	+	+		+		

...Table 2. Contd.

Table 2. Contd...

Taxon	CCA species code	Intertidal sandflat		Mangrove site		Port site	
		CONT	P	CONT	P	CONT	P
<i>Fragilariopsis</i> (Hustedt in Schmidt) sp.	FragS	+	+				
<i>Glyphodesmis</i> (Greville) sp?	Glys?			+	+		
<i>Grammatophora marina</i> (Lyngbye) Kutzing	GraM	+	+				
<i>Gyrosigma balticum</i> (Ehrenberg) Cleve	GyB			+	+		
<i>Gyrosigma wansbeckii</i> (Donkin) Cleve	GyW			+	+		
<i>Gyrosigma</i> (Hassall) sp.	Gys			+	+		
<i>Mastogloia rostrata</i> (Wallich) Hustedt	MasR		+				
# <i>Navicula crucicula</i> (W. Smith) Donkin	NvC	+	+				
<i>Navicula directa</i> (W. Smith) Ralfs in Pritchard	NvD	+		+			
<i>Navicula granii</i> (Jorgensen) Gran	NvG	+					
<i>Navicula subinflata</i> Grunow	NvS				+		
<i>Navicula transitans</i> var. <i>derasa</i> (Grunow, in Cleve and Grunow) Cleve	NvTDs	+	+	+	+	+	+
<i>Navicula transitans</i> var. <i>derasa</i> f. <i>delicatula</i> Heimdal	NvTDe	+	+	+	+		
<i>Navicula vanhoeffenii</i> Gran	NvV	+					
<i>Navicula</i> (Bory) sp.	Nvs	+	+	+	+	+	+
<i>Nitzschia panduriformis</i> Gregory	NiP			+		+	
<i>Nitzschia seriata</i> Cleve	NiS	+				+	
<i>Nitzschia sigma</i> (Kutzing) W. Smith	NiSg			+		+	+
<i>Nitzschia</i> (Hassall) sp.	Nis	+	+	+	+		
<i>Pleurosigma elongatum</i> W. Smith	PIEl			+	+		
<i>Pleurosigma fasciola</i> (Ehrenberg) W. Smith	PIF			+	+		
<i>Pleurosigma parkeri</i> Harrison	PIPa			+			
<i>Pleurosigma</i> (W. Smith) sp.	Pls			+	+		
<i>Pseudo-nitzschia lineola</i> (Cleve) Hasle	PsnL					+	
<i>Pseudo-nitzschia</i> (H. Peragallo) sp.	Psns	+		+	+		
<i>Thalassionema bacillare</i> (Heiden in Heiden & Kolbe) Kolbe	ThnB					+	+
<i>Thalassionema frauenfeldii</i> (Grunow) Hallegraeff	ThnF			+			
<i>Thalassionema nitzschioides</i> (Grunow) Mereschkowsky	ThnN	+	+	+	+	+	+

CONT, f/2(MBL); P, penicillin; + indicates presence of the diatom species.

* From 2009, the genus *Amphora* has been modified to *Halamphora* by Levkov (2009).

The currently accepted name of *Navicula crucicula* (W. Smith) Donkin is *Parlibellus cruciculus* (W. Smith) Witkowski, Lange-Bertalot & Metzeltin 2000.

Table 3. Species richness (d), evenness (J') and diversity (H') of the viable, benthic diatom assemblages in control and penicillin treatments at the study sites during the four sampling periods

	Species richness (d)		Species evenness (J')		Species diversity (H')	
	CONT	P	CONT	P	CONT	P
<i>(a) Intertidal sandflat</i>						
PM-I	1.81 (±0.17)	1.56 (±0.26)	0.70 (±0.06)	0.61 (±0.12)	1.86 (±0.19)	1.42 (±0.32)
PreM	1.71 (±0.14)	0.95 (±0.19)	0.81 (±0.07)	0.44 (±0.26)	2.07 (±0.07)	0.83 (±0.43)
Mon	0.74 (±0.08)	0.49 (±0.42)	0.69 (±0.01)	0.61 (±0.53)	1.05 (±0.08)	0.84 (±0.73)
PM-II	1.57 (±0.43)	0.76 (±0.59)	0.56 (±0.30)	0.44 (±0.27)	1.41 (±0.69)	0.62 (±0.49)
<i>(b) Mangrove site</i>						
PM-I	1.99 (±0.25)	0.90 (±0.16)	0.57 (±0.10)	0.82 (±0.05)	1.61 (±0.24)	1.31 (±0.22)
PreM	1.16 (±0.37)	0.71 (±0.20)	0.67 (±0.05)	0.88 (±0.11)	1.39 (±0.30)	1.25 (±0.19)
Mon	2.41 (±0.04)	2.10 (±0.79)	0.50 (±0.04)	0.74 (±0.08)	1.45 (±0.08)	1.85 (±0.22)
PM-II	1.33 (±0.26)	0.61 (±0.33)	0.58 (±0.06)	0.75 (±0.17)	1.30 (±0.16)	0.97 (±0.53)
<i>(c) Port site*</i>						
PM-I	0.95 (±0.31)	-	0.66 (±0.30)	-	0.97 (±0.13)	-
PreM	1.00 (±0.24)	0.70 (±0.20)	0.50 (±0.07)	0.68 (±0.17)	0.93 (±0.05)	0.93 (±0.09)
Mon	1.80 (±0.48)	1.25 (±0.39)	0.85 (±0.04)	0.83 (±0.10)	1.98 (±0.26)	1.66 (±0.16)
PM-II	-	-	-	-	-	-

CONT, f/2(MBL); P, penicillin; PM-I: post-monsoon I; PreM: pre-monsoon;

Mon: monsoon; PM-II: post-monsoon II.

Average and standard deviation values in brackets are provided.

* No species observed in PM-I/P and a single species reported in PM-II/CONT and P.

Table 4. Culturable bacteria in the control and penicillin treatments at the study sites during the four sampling periods

Sampling period	Intertidal sandflat		Mangrove site		Port site	
	CONT	P	CONT	P	CONT	P
<i>(a) PM-I</i>						
<i>Alcaligenes</i>	+			+	+	+
<i>Bacillus</i>			+			
<i>Corynebacterium</i>			+			
<i>Enterobacteriaceae</i>	+					
<i>Micrococcus</i>					+	
<i>Pseudomonas</i>	+	+		+		
<i>Vibrio</i>		+				
<i>(b) PreM</i>						
<i>Alcaligenes</i>	+	+	+	+	+	+
<i>Bacillus</i>					+	
<i>Corynebacterium</i>			+			+
<i>Micrococcus</i>			+			
<i>Pseudomonas</i>	+	+		+		
<i>(c) Mon</i>						
<i>Alcaligenes</i>	+			+		
<i>Bacillus</i>	+		+	+	+	
<i>Corynebacterium</i>			+		+	+
<i>Pseudomonas</i>	+	+				+
<i>Vibrio</i>		+				
<i>(d) PM-II</i>						
<i>Alcaligenes</i>	+	+	+		+	+
<i>Bacillus</i>	+			+		
<i>Corynebacterium</i>					+	
<i>Micrococcus</i>					+	
<i>Pseudomonas</i>	+		+	+		+
<i>Vibrio</i>		+				

CONT, f/2(MBL); P, penicillin; PM-I: post-monsoon I; PreM: pre-monsoon;

Mon: monsoon; PM-II: post-monsoon II.

+ indicates presence of the bacterial species. Bacteria were identified based on Bergey's Manual of Systematic Bacteriology by Holt *et al.* (1994).