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The effect of bacteria on diatom community structure – the ‘antibiotics’ approach

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Running head: The effect of bacteria on diatom community structure

Abstract

To investigate the effect of bacteria on diatoms at the community level, sediment samples from an intertidal tropical environment were treated with penicillin (a β -lactam antibiotic that can affect diatoms only through bacteria). Streptomycin (an aminoglycoside) and chloramphenicol, antibiotics that can potentially affect protein synthesis in diatom organelles and photosynthesis, were also used for comparison. The changes in diatom community structure and the resistant and tolerant bacterial fractions were analyzed through microscopy, culture techniques and Denaturing Gradient Gel Electrophoresis. The reduction in bacterial abundance when treated with penicillin resulted in suppression of *Amphora coffeaeformis*, a dominant diatom in the study area. The bacterial community preferred the ‘tolerance’ strategy over ‘resistance’ in response to treatment with penicillin; these changes in bacterial dynamics were probably linked to the concurrent changes in diatom community structure. The observations with penicillin differed from those with streptomycin that did not seem to affect diatoms significantly, and chloramphenicol, which inhibited diatoms consistently. Overall, the results of this study highlight the significance of bacteria in structuring benthic diatom communities, and call for the inclusion of the ‘antibiotics’ approach, in studies addressing diatom-bacterial interactions at the community level.

Keywords: antibiotics; diatoms; bacteria; penicillin; *Amphora coffeaeformis*

Abbreviations: CONT: f/2(MBL); P: penicillin; C: chloramphenicol; PSC: antibiotic combination; 3S, 2S, S, S/10: streptomycin treatments (different concentrations); DGGE: Denaturing Gradient Gel Electrophoresis

1. Introduction

Diatoms share a close but ambiguous relationship with bacteria. In addition to being symbiotic, diatom-bacterial interactions may also be antagonistic (Croft et al., 2005; Grossart and Simon, 2007). Diatoms and bacteria might support each other through cross-feeding, possibly optimized by exchange of chemical factors (Bruckner et al., 2008). The advantages of this intrinsic association with bacteria are evident in the high growth rate and enhanced Extracellular Polymeric Substances (EPS) production of non-axenic diatoms compared to axenic diatoms (Patil and Anil, 2005) and in the superior tolerance of *Amphora coffeaeformis* to copper and tributyltin in the presence of bacteria (Thomas and Robinson, 1987). Bacteria also influence various processes mediated by diatoms such as production of exudates and capsule formation, which modulate the growth and metabolic activities of diatoms (Bruckner et al., 2008). Bacteria influence the strength by which diatoms adhere to a substratum (Wigglesworth-Cooksey and Cooksey, 2005), and sediment stabilization by diatoms (Gerbersdorf et al., 2008; Wigglesworth-Cooksey et al., 2001).

Though a lot of information is available about the relevance of bacteria to diatoms and vice-versa, not much is known about the effect of bacteria on diatoms, at the community level. To investigate this aspect, we incubated sediment samples with penicillin (a β -lactam antibiotic). Penicillin inhibits cell wall peptidoglycan synthesis in bacteria and is effective mainly against gram-positive bacterial strains (Josephson et al., 2000). It cannot directly affect diatoms that have a different cell wall structure. Thus, any effects on diatom community structure when treated with penicillin, represents the effect of bacteria. Streptomycin (an aminoglycoside) and chloramphenicol, with differing modes of action, were used for comparison. Both these antibiotics inhibit protein synthesis, but by binding to different ribosomal subunits. Streptomycin binds to the 30S ribosomal subunit whereas chloramphenicol binds to the 50S ribosomal subunit (Josephson et al. 2000). Both these antibiotics may affect diatoms directly, through inhibition of protein synthesis in diatom organelles (70S) (Cullen and Lesser, 1991; Stanier et al., 1971). Among the two, chloramphenicol is known to inhibit photosystem II in diatoms (Ohad et al., 1984).

In addition to analyzing the changes in the diatom community, we focused on bacteria resistant and tolerant to antibiotics and the total bacterial community. The resistant bacterial fraction can tolerate and multiply in the presence of the particular antibiotic, whereas the tolerant bacterial fraction represents those bacteria that can tolerate but not divide in the presence of the antibiotic. The tolerant bacterial

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).

In view of the above, this study addresses the following questions. (1) What are the changes in diatom communities when bacteria are suppressed by penicillin? (2) Are these changes different from those of chloramphenicol, streptomycin and the antibiotic combination effects? (3) What are the changes in the antibiotic-resistant and -tolerant bacterial fractions?

2. Methods

2.1. Sampling

Sampling was carried out in May 2008 on a sand flat at Dias Beach (15°27' N; 73° 48' E) located near Dona Paula Bay and surrounded by the Mandovi and Zuari estuaries. This beach is approximately 200 m in length and is bounded on both sides by rocky cliffs. The benthic 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.

2.2. Experimental design

The extinction-dilution method (most-probable-number method, MPN) (Thronsen, 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 autoclaved, artificial seawater (MBL) (Cavanaugh, 1975) was used as the control diluent (CONT). Nutrient-enriched f/2 medium prepared in autoclaved, 0.45 µm-filtered, aged seawater [f/2(ASW)] was used as an additional control for comparison. Usually, f/2(ASW) is used in most studies. However, its chemical composition varies depending on the batch of the ASW used and the duration of the ageing period. Therefore, in this study, f/2(MBL) having a defined chemical composition, was used as the main control diluent, and will henceforth be termed CONT.

Penicillin, streptomycin and chloramphenicol were added to CONT at the following concentrations: 0.2 mg ml⁻¹ (penicillin-P), 0.1 mg ml⁻¹ (streptomycin-S) and 0.02 mg ml⁻¹ (chloramphenicol-C)

individually and in combination (PSC) and represented ‘qualitative’ effects. Additional streptomycin concentrations [0.3 mg ml^{-1} (3S), 0.2 mg ml^{-1} (2S), and 0.01 mg ml^{-1} (S/10)] were also used, since incubation of sediment samples with 0.1 mg ml^{-1} streptomycin enhanced diatom growth (approximately 133% compared to control values) in experiments conducted separately (personal observations). These streptomycin treatments reflected ‘quantitative’ effects. The antibiotic concentrations used in this study are in the range used to make diatom cultures axenic (Patil and Anil, 2005).

Subsequently, diatoms and resistant and tolerant bacteria were analyzed in triplicate. The bacterial community was also subjected to Denaturing Gradient Gel Electrophoresis (DGGE) profiling.

2.3. Analysis of diatoms

Sediment (1 g) was suspended in each diluent at a concentration of $0.1 \text{ g sediment ml}^{-1}$. Ten-fold dilutions up to 10^{-5} were carried out, followed by inoculation of 1 ml aliquots from each dilution into five replicate culture wells. The culture wells were incubated at $25 \pm 1 \text{ }^\circ\text{C}$ with a 12:12 h light : dark cycle for 7 days. Subsequently, 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 of diatoms in the sediment sample (MPN g^{-1} wet sediment) was calculated by counting the positive wells in three consecutive dilutions and interpreting that value using the statistical MPN table provided by Thronsdon (1978). The 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.

2.4. Quantification of antibiotic-resistant and -tolerant bacteria

Aliquots of 0.1 ml (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. ZMA 2216 without antibiotics was also used; the difference in bacterial counts on both media represented the antibiotic-tolerant bacteria. Both resistant and tolerant bacteria are expressed as CFU g^{-1} wet sediment.

2.5. 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 PowermaxTM 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). A band pattern was generated by using a band finding algorithm available with the software, and by manually examining the gel to match the bands that were not identified by the software.

2.6. Data analyses

Normality and homogeneity of variances were determined using Shapiro's and Levene's tests respectively. Diatom abundance data fulfilled the assumptions of normality and were therefore analyzed for significant differences between (1) f/2(ASW) and CONT using the paired t-test, and (2) all treatments through one-way ANOVA. The post-hoc Dunnett test was carried out to determine which of the treatments varied significantly from CONT. The total, resistant and tolerant bacterial fractions data failed the requirements for parametric analysis, and were thus analyzed through non-parametric Kruskal-Wallis tests. All these analyses were carried out using the STATISTICA 8 software.

Univariate measures of the diatom community, i.e., Margalef's species richness (d), Pielou's evenness (J') and Shannon-Wiener's diversity index (H'), were calculated. Diatom abundance data and

the DGGE band pattern were converted into a lower triangular similarity matrix using Bray- Curtis coefficients and subjected to cluster analysis by the group average method and ordination by non-metric multidimensional scaling (NMDS). Prior to these analyses, diatom abundance data was fourth root ($\sqrt[4]{\cdot}$)-transformed whereas the DGGE band pattern was transformed into a presence/absence matrix using Quantity One software by BioRAD. These analyses were done using PRIMER software version 5.

3. Results

3.1. Diatom communities

CONT supported higher average diatom abundance (1.16×10^3 diatoms cm^{-3} wet sediment) compared to f/2(ASW) (7.78×10^2 diatoms cm^{-3} wet sediment) (Fig. 1). Yet, no significant difference in diatom abundance was observed in these treatments (paired t-test, $p=\text{ns}$).

The P treatment supported $7(\pm 3)\%$ diatom abundance compared to CONT (Fig. 1). Diatom abundance varied significantly across all the treatments (one-way ANOVA, $p=0.003$) and ranged from $3(\pm 2)\%$ to $96(\pm 88)\%$ in the ‘qualitative’ treatments (P, S, C and PSC) and from $53(\pm 31)\%$ to $96(\pm 88)\%$ in the ‘quantitative’ streptomycin treatments (Fig. 1). The post-hoc Dunnett test revealed that the PSC ($p=0.001$), P ($p=0.002$) and C ($p=0.010$) treatments were significantly different from CONT. The streptomycin treatments that did not show a linear decrease in diatom abundance with increasing concentration (Fig. 1), did not contribute to the statistically significant differences across the treatments (Dunnett post-hoc test, $p=\text{ns}$ for 3S, 2S, S and S/10).

Diatoms belonging to 8 genera were recorded in all the treatments (Table 1). A comparison of the diatoms emerging in CONT and f/2(ASW) indicated that *Fragilariopsis* sp., *Navicula transitans* var. *derasa*, *Navicula vanhoeffennii* and *Synedropsis hyperborea* were recorded only in CONT whereas *Navicula directa* was recorded only in f/2(ASW) (Table 1). *Navicula* sp. was most abundant in all the treatments (Fig. 2). *Amphora rostrata*, *A. coffeaeformis*, *Fragilariopsis* sp., *Navicula crucicula* and *N. transitans* var. *derasa* f. *delicatula* were recorded in all the treatments except PSC (Table 1, Fig. 2). *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 (Table 1, Fig. 2).

The changes in diatom community structure were reflected in species richness, diversity and evenness measures (Fig. 3). Compared to CONT, the diatom community in f/2(ASW) had lower Margalef's species richness (d), Pielou's evenness (J') and Shannon-Wiener diversity (H') (Fig. 3). 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. 3).

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. 4a). Sub-group A consisted of all the streptomycin treatments and CONT, with abundance ranging from 538-1162 diatoms cm^{-3} wet sediment (Fig. 1). The treatments belonging to this group were dominated by *Navicula* sp., *A. coffeaeformis* and *A. rostrata* (Fig. 2). Sub-group B consisted of f/2(ASW) and the C treatment, having similar species distribution (Fig. 2) and with abundance ranging from 267-778 diatoms cm^{-3} wet sediment (Fig. 1). PSC with lowest diatom abundance (47 diatoms cm^{-3} wet sediment) (Fig. 1), species richness (d) and diversity (H') (Fig. 3) was the most dissimilar entity. P, with 80 diatoms cm^{-3} wet sediment (Fig. 1), was the second dissimilar treatment (Fig. 4a). The dissimilar treatments (PSC and P) were clearly evident in the NMDS ordination (Fig. 4b).

3.2. Bacterial communities

Abundance of bacteria in CONT averaged 4.43×10^9 CFU g^{-1} wet sediment and was similar to bacterial abundance in f/2(ASW) (Fig. 5). No significant differences in the bacterial abundance in both these treatments were observed (Kruskal-Wallis test, $p=\text{ns}$).

Resistant bacteria ranged from 5.33×10^6 - 3.43×10^9 CFU g^{-1} wet sediment (Fig. 5a) whereas tolerant bacteria ranged from 1.38×10^6 - 2.58×10^9 CFU g^{-1} wet sediment (Fig. 5b). Both, resistant and tolerant bacteria varied significantly across all the treatments (Kruskal-Wallis tests, $p=0.047$ and $p=0.033$ for resistant and tolerant bacteria respectively) and were suppressed to the maximum extent in the antibiotic combination and the streptomycin treatments. Tolerant bacteria were more abundant compared to resistant bacteria in all the treatments except C, PSC and S/10 (Fig. 5a,b).

In P, resistant and tolerant bacterial fractions peaked to maximum values (approx. 20% and 47% of control values respectively) on day 7 (Fig. 6). In C, both groups increased explosively to 2,500% and

1000% respectively on day 4. The resistant bacteria peaked again on day 6. In PSC, tolerant bacteria were observed on day 1 whereas resistant bacteria were detected only on day 4. In the streptomycin treatments, resistant and tolerant bacteria were <10% of the corresponding control values in 3S, 2S and S and comparatively higher in S/10 (Fig. 6).

Cluster analysis of the day 7 bacterial community in all the treatments analyzed through DGGE indicated 3 groups (Fig. 7a). 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. 7a); their distinctness is apparent in the NMDS ordination (Fig. 7b).

4. Discussion

The CONT diluent supported higher diatom abundance and similar bacterial profiles compared to f/2(ASW). These differences in diatom abundance (though not statistically significant) (Fig. 1) and species composition in both treatments (Fig. 2), could probably be linked to the composition of the ASW, which unlike the CONT diluent, could contain substances that influence diatom metabolism, for e.g., chelators, growth promoters/ inhibitors.

Incorporation of penicillin in the control diluent resulted in the most dissimilar diatom community among all the individual antibiotic treatments. This was reflected in the post-hoc Dunnett test which indicated that the penicillin treatment was statistically the most different from CONT, among all the individual antibiotic treatments. This observation in addition to the marked reduction in diatom abundance (upto 7% of CONT values) and species richness, when treated with penicillin, highlights the relevance of bacteria to diatoms. This is because penicillin cannot affect diatoms directly as it inhibits peptidoglycan synthesis mainly in Gram positive bacteria. Diatoms have a different cell wall structure (Tomas, 1997) and therefore cannot be directly affected by penicillin.

The bacteria suppressed by penicillin and consisting mainly of Gram-positive bacteria, appear 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 P treatment (Table 1; Fig. 4). *A. coffeaeformis* is a fast reproducer and has strong competitive potential (Mitbavkar and Anil, 2007). It is known to produce domoic acid, a water-soluble toxin (Maranda et al., 1989), that provides a competitive advantage to the organism, compared to the non-toxin producing forms. Even when present with *N.*

transitans var. *derasa* f. *delicatula* at a ratio of 1:99, it can overtake its competitor (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 Cu and tributyltin than non-axenic cultures (Thomas and Robinson, 1987). Our results 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 P treatment was most similar to the PSC treatment, in terms of extent of inhibition of diatoms (apparent in the cluster analysis). The inhibition of most diatoms in PSC (upto 3% of control) 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 (Josephson et al., 2000). 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.

Marked changes in diatom abundance were also observed in the other antibiotic treatments. Streptomycin and chloramphenicol, though having the potential to affect diatoms directly (Stanier et al., 1971), affected diatoms differentially (Figs. 1,2). Abundance of most diatoms was reduced in chloramphenicol. Yet, the species composition of the diatom community in the chloramphenicol treatment mirrored that in f/2(ASW), and was reflected in the cluster analysis (Fig. 4). However, streptomycin did not seem to affect the diatom community. This is clear from the following trends - (1) Diatom abundance ranged from 53 - 96% in the streptomycin treatments and did not show a dose-dependent trend (Fig. 1), (2) The post-hoc Dunnett test indicated no significant differences in all the streptomycin treatments compared to CONT, (3) *Amphora* and *Navicula* grew abundantly in the streptomycin treatments (Fig. 2) and (4) *Cocconeis* sp., *N. transitans* var. *derasa* and *Thalassiosira* spp. occurred in only the streptomycin treatments (Table 1). These characteristics may be responsible for the similarity of the streptomycin treatments with CONT, reiterated by the cluster and statistical analyses.

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. In case of penicillin that inhibits diatoms to the maximum extent among all the individual antibiotic treatments, tolerance seems to be preferred over resistance. The resistant bacterial fraction has already passed through a genetic bottleneck, and thus has reduced functional diversity, so-called loss of fitness (Anderson, 2005). Compared to this, the tolerant bacteria are characterized by the ability to adapt rapidly to antibiotic stress, for e.g., by switching on/off genes linked to general stress responses (Gefen and Balaban, 2009). In fact, the strategy of tolerance, i.e., surviving in the non-multiplying state, is particularly relevant in the case of antibiotics that act on growing cells, for e.g., β -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). This ‘tolerance’ strategy of the bacterial community appears to be the most suitable in the context of penicillin.

Characteristic shifts in the dynamics of resistant and tolerant bacteria have also been observed in the other antibiotic treatments (Fig. 6). For e.g., PSC-resistant bacteria seem to require an extended lag period (upto 4 days) to cope with the presence of all 3 antibiotics in combination. In C, resistant and tolerant bacteria increased explosively after a 3 day lag period. However, this increase was not sustained till the end of the incubation period, indicating the sustained effectiveness of the chloramphenicol concentration used.

In the streptomycin treatments, both resistant and tolerant bacteria were suppressed to <10%, with the exception of tolerant bacteria in S/10 (Fig. 6). Given that the diatom communities in the streptomycin treatments were similar to that under control conditions, the disparity in the abundance profiles of diatoms and bacteria suggest that the bacteria suppressed by streptomycin were not essential for diatom growth. These shifts in the dynamics of resistant and tolerant bacteria, 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. In this context, the results with penicillin, i.e., the preference of the bacterial

community for tolerance over resistance could be indicative of the physiological/functional shifts in bacterial communities and may probably be responsible for the changes in diatom community structure when treated with penicillin.

In the C and PSC treatments, resistance was preferred over tolerance (Fig. 5a,b). This similarity in the C and PSC treatments was reflected in the cluster and ordination analyses of the bacterial community based on DGGE band profiles (Fig. 7). It however differed from the cluster and ordination analyses of the treatments based on diatoms (Fig. 4a), which indicated that the C and f/2(ASW) treatments supported similar diatom communities. These differences in the cluster and ordination patterns of the diatom and bacterial communities could probably be influenced by the differential factors regulating diatom and bacterial communities and need to be evaluated in detail.

Summarizing, the ‘antibiotics’ approach used to determine the effect of bacteria on diatom community structure revealed that benthic 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 resistant and tolerant bacteria and were different from the potentially direct effects of streptomycin and chloramphenicol. These observations illustrate not only the significant role of bacteria in regulating diatom communities in benthic environments, but also the utility of the ‘antibiotics’ approach to investigate the relationships between bacteria and diatoms at the community level. To our knowledge, this is the only method so far that allows the experimental manipulation of the bacterial community in natural samples, followed by subsequent analysis of the effects on the diatom community. The level of bacterial suppression can also be controlled by manipulating the concentration of penicillin used.

This ‘antibiotics’ approach also has a very important tangential benefit. It can be used to study the effects of antibiotics on diatom communities, the base of aquatic food webs. This is pertinent in view of the prevalence of antibiotics in natural environments, attributed to anthropogenic sources, and also due to the varying ecological roles of antibiotics (ranging from competitive processes to signaling mechanisms) [Martinez (2008) and references therein]. Given these advantages (both direct and applied), the incorporation of this approach in studies on diatom-bacterial interactions at the community level is strongly advocated.

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Legends to figures

Fig. 1. Diatom abundance (cm^{-3} wet sediment) 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. Mean \pm SD values are shown. $n=3$. The values in the bracket indicate percentage values compared to CONT. * indicates the treatments that are significantly different from CONT; a, b, c indicates their ranking (descending order) in a post-hoc Dunnett test, done after a one-way ANOVA. *a = most significantly different from CONT.

Fig. 2. Species composition (%) of the diatom communities 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. Minor species represent the sum of the species contributing $<2\%$.

Fig. 3. Univariate measures. (a) Margalaf' 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. Mean values are shown.

Fig. 4. (a) Cluster dendrogram and (b) non-metric multidimensional scaling (NMDS) ordination of the different treatments based on the diatom community using Bray-Curtis similarity coefficients. 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.

Fig. 5. Abundance of (a) resistant bacteria and (b) tolerant bacteria in the different treatments on day 7, expressed as CFU g^{-1} wet sediment. $n=3$. 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. Mean \pm SD values are shown. Kruskal-Wallis tests indicated significant differences between the treatments, (a) $p=0.047$, (b) 0.033 .

Fig. 6. Abundance of resistant and tolerant bacteria in the different antibiotic treatments throughout the incubation period, expressed as percentage of respective control values. $n=3$. Antibiotic treatment details are provided in the text. Mean \pm SD values are shown.

Fig. 7. (a) Cluster dendrogram and (b) non-metric multidimensional scaling (NMDS) ordination of the different treatments based on the bacterial community DGGE profile using Bray-Curtis similarity coefficients. 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.

Table 1
Diatoms observed in the different treatments.

Taxa	f/2(ASW)	CONT	P	C	PSC	3S	2S	S	S/10
<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>Melosira</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 vanhoeffennii</i> Gran		+							
<i>Navicula</i> spp.	+	+	+	+	+	+	+	+	+
<i>Nitzschia</i> spp.	+	+		+		+	+	+	+
<i>Synedropsis hyperborea</i> (Grunow) Hasle, Medlin & Syvertsen		+						+	+
<i>Thalassiosira</i> spp.	+	+				+	+	+	+
Unidentified diatoms	+	+	+	+	+	+	+	+	+

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.

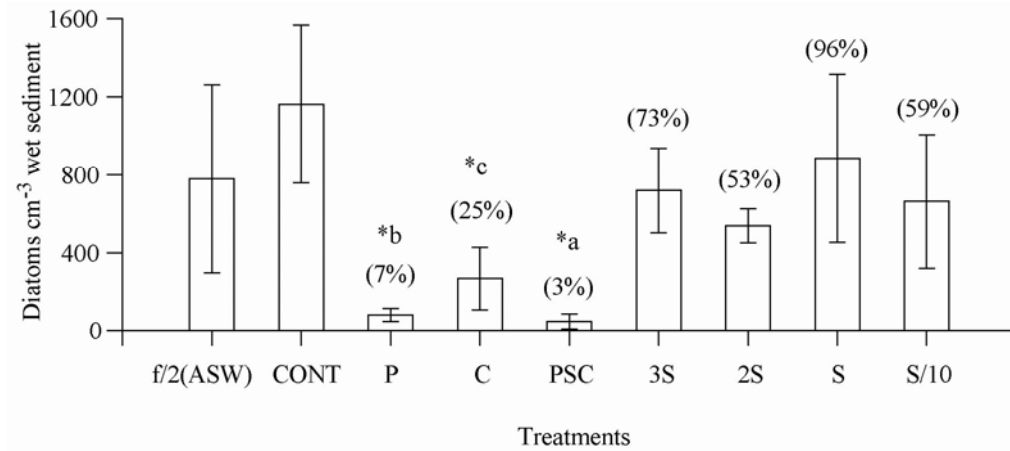


Fig. 1

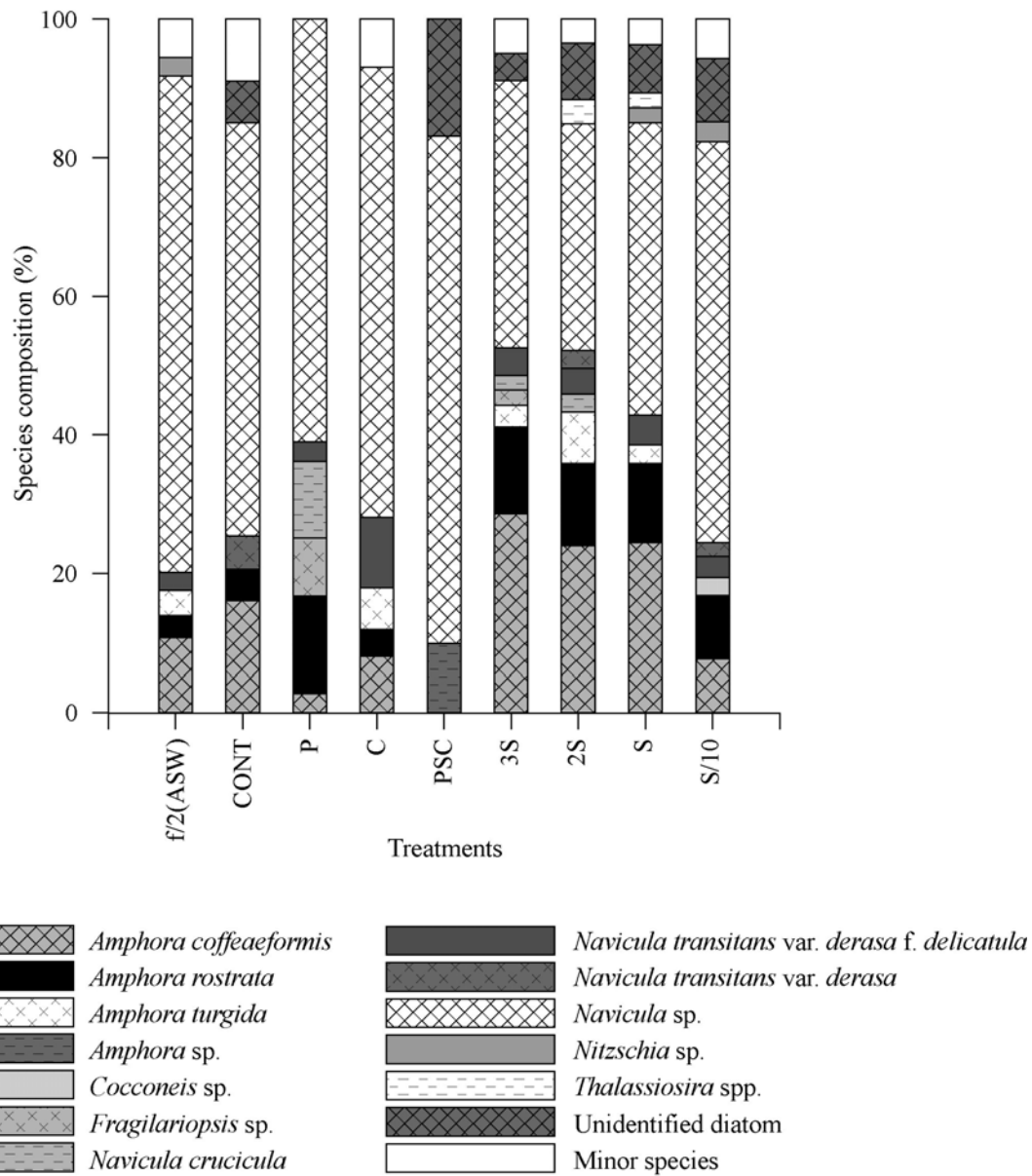


Fig. 2

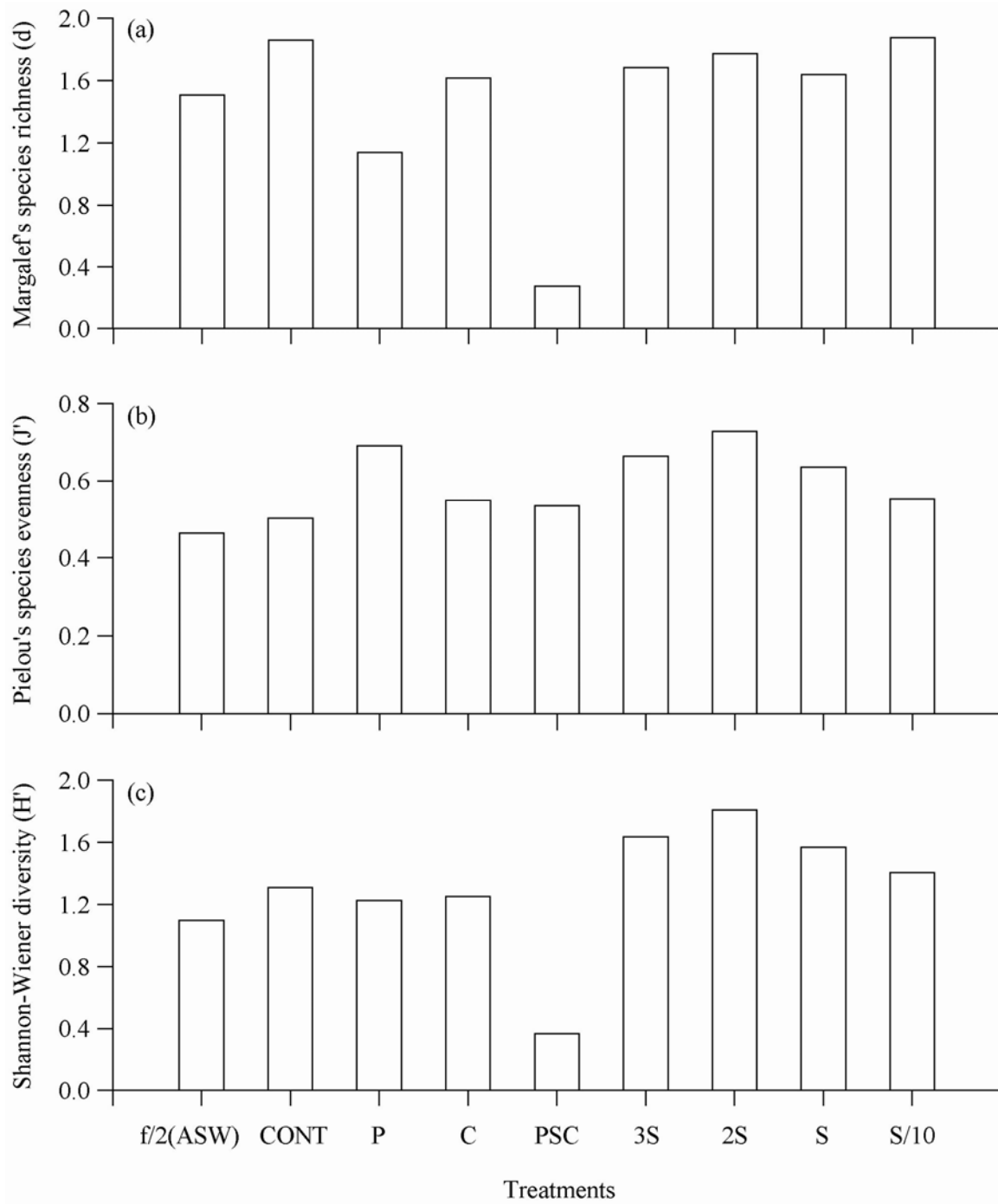


Fig. 3

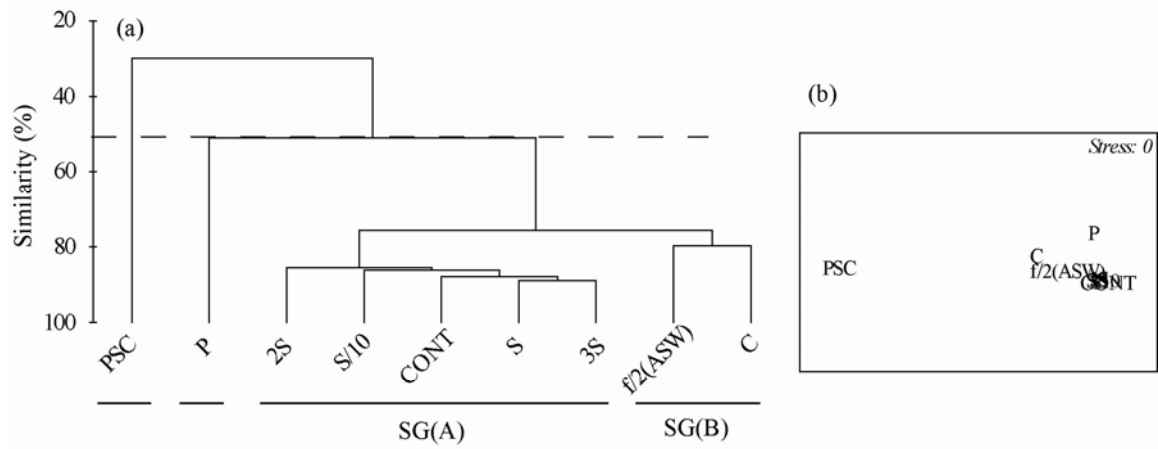


Fig. 4

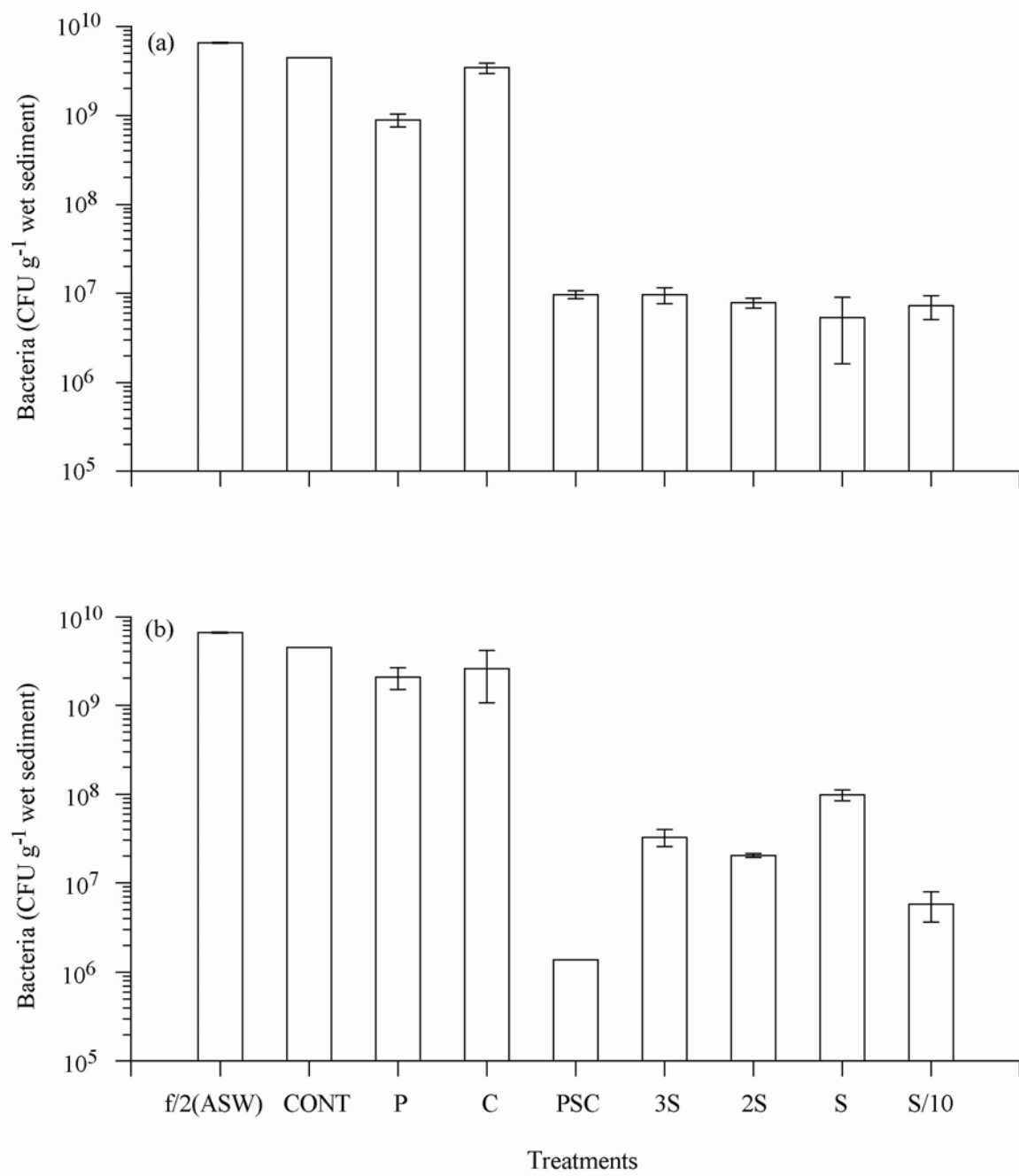


Fig. 5

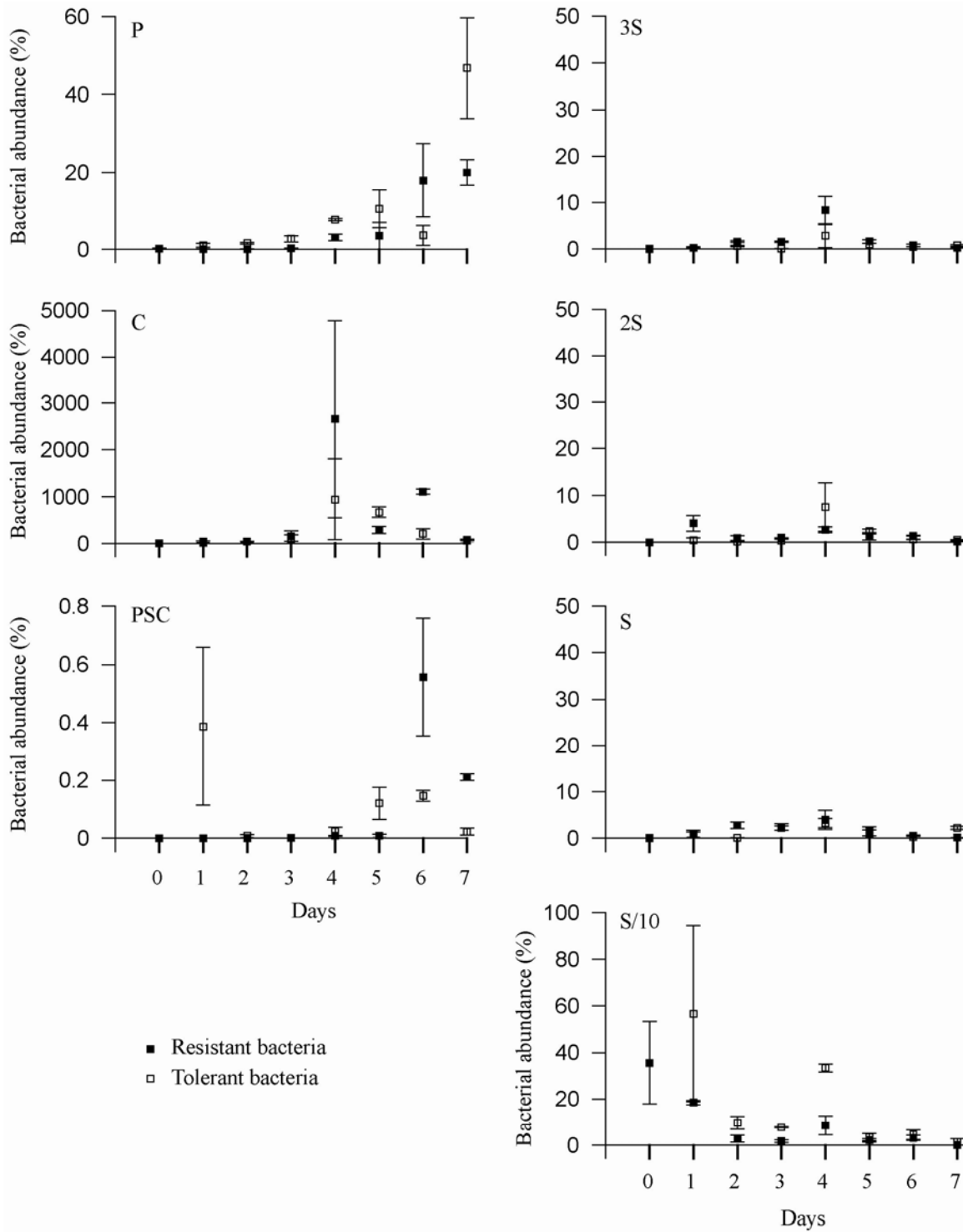


Fig. 6

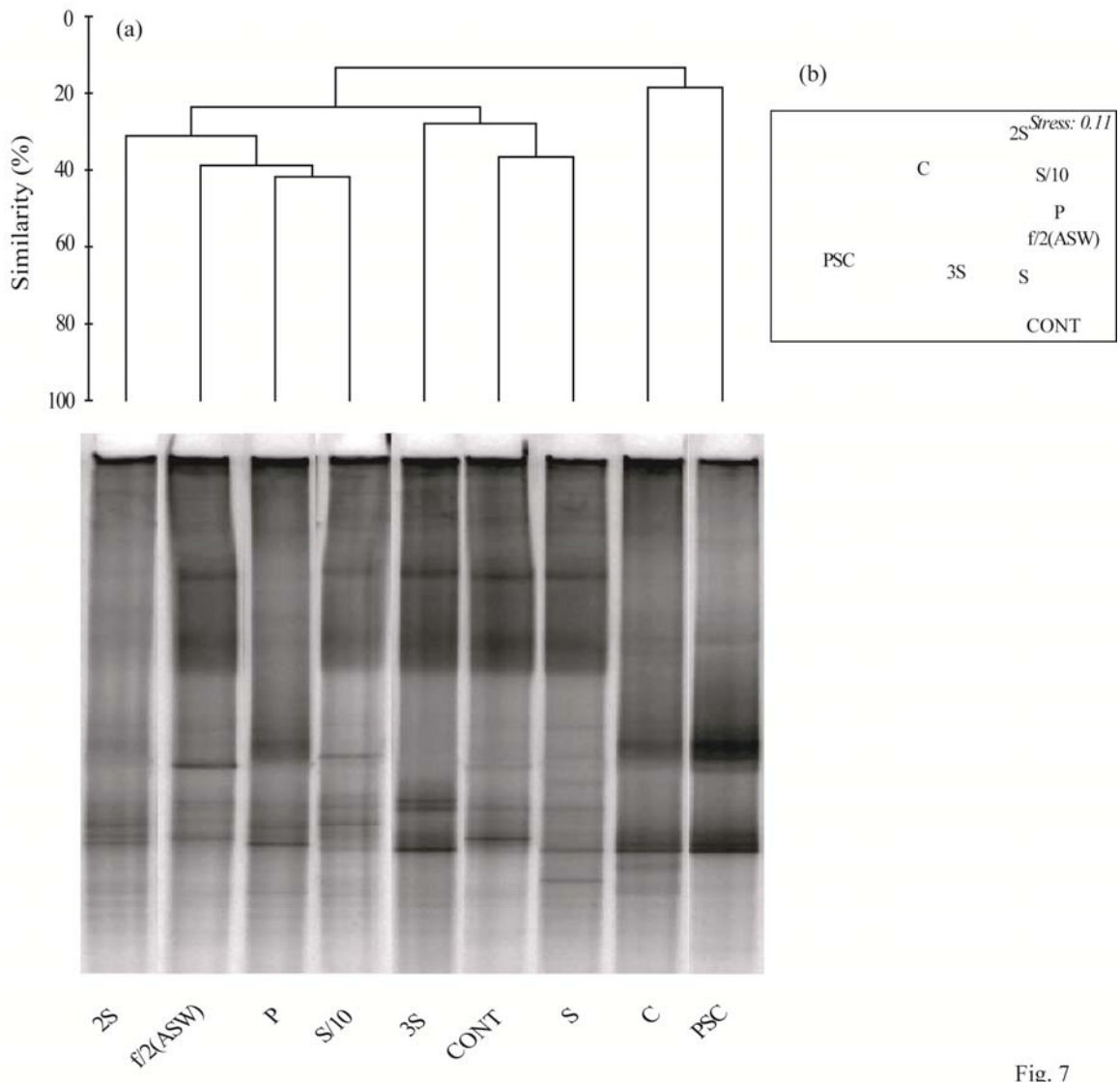


Fig. 7