Influence of diatom exopolymers and biofilms on metamorphosis in the barnacle *Balanus amphitrite*

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ABSTRACT: Natural biofilms constitute a complex network of microorganisms (bacteria, diatoms, protozoa, fungi) and their extracellular polymeric substances (EPS), which influence settlement in benthic invertebrates. We investigated the influence of diatom (Bacillariophyceae) films and EPS (>1000 molecular weight) on metamorphosis in the acorn barnacle *Balanus amphitrite* Darwin, a dominant fouling organism, using axenic and non-axenic films, and free and biofilm EPS, of 5 species of pennate diatoms: *Amphora coffeaeformis* and *A. rostrata*, and *Navicula transitans* var. *derasa* f. *delicatula*, *N. crucicula* and *N. subinflata*. Fourier-transformed infrared spectroscopy (FTIR) spectra revealed that the EPS produced by diatoms are of similar nature; however, the proportion of mono-saccharides varies with species, indicating a potential role for influencing larval metamorphosis. Free EPS of axenic diatoms had no effect, whereas biofilm EPS induced larvae to metamorphose. *Amphora* spp. produced more biofilm EPS, but had relatively weaker effects than *Navicula* spp. Axenic diatom films also facilitated larval metamorphosis (depending on species and cell density) in some cases, suggesting that the cues provided by the diatoms themselves can also mediate invertebrate larval metamorphosis in the absence of microbial films. Non-axenic diatom films (which had higher cell densities) and biofilm EPS promoted metamorphosis to a greater degree than those of axenic diatoms. Enhancement of metamorphosis depended on diatom species and on their density in the films, as well as on the composition of their EPS. Differential responses of barnacle larvae to different diatom species and their EPS indicate that each diatom species provides a different set of physico-chemical signals to settling larvae.

KEY WORDS: Diatoms · Bacillariophyceae · Extracellular polymeric substances · Biofilms · Larvae · *Balanus amphitrite*

INTRODUCTION

A large number of sessile invertebrates produce pelagic larvae as part of their life history. The final stage of the larvae plays a crucial part in their life cycle, as its sole purpose is to search and attach to the most suitable site on the substratum for further development into sedentary juvenile individuals. The commencement of larval settlement and metamorphic processes are triggered by environmental (physical and biological) stimuli. Several studies have suggested that biofilms influence surface exploration and substratum choice of invertebrate larvae and can play an important role in their settlement and metamorphosis (e.g. Keough & Raimondi 1996, see below). Biofilm is a complex network of microorganisms (bacteria, diatoms,

protozoa and fungi) and their extracellular polymeric substances (EPS). Amongst these, diatoms are the earliest eukaryotic colonizers of submerged surfaces and one of the most conspicuous organisms in biofilms. Attachment of diatoms is invariably associated with the production of EPS in the form of pads, envelopes, stalks or tubes (Daniel et al. 1987, Hoagland et al. 1993). Exopolysaccharides appear to be involved in many functions in the adherent biofilms, including motility, protection and maintenance (Characklis & Cooksey 1983, Hoagland et al. 1993). These EPS are composed of polysaccharides with variable amounts of proteins, and they either contribute to the biofilm matrix (Cooksey 1992) or are released by the microorganisms to the surrounding medium as free EPS (Sutherland 1985, Khandeparker & Bhosle 2001).

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The formation of biofilm on a substratum modifies the surface chemistry (Characklis & Cooksey 1983) and strongly influences larval recruitment to that surface, which leads to economic problems in the shipping industry and in industrial aquatic processes. Marine invertebrate larvae are presented with a wide range of cues as they approach a substratum. These cues may be physical or biologically derived chemical ones associated with bacteria, microflora and microfauna and can affect larval attachment (Maki et al. 1992). Microbial biofilms have generally been examined as a stimulus for the settlement of macrofouling organisms (Crisp 1974). The larvae may use specific chemical signatures from biofilms or characteristic microbial assemblages to indicate preferred ecological conditions at a site. Extensive relevant literature reports that biofilms can have varying effects on invertebrate larval metamorphosis; e.g. barnacles (Avelin Mary et al. 1993, O'Connor & Richardson 1996, Anil & Khandeparker 1998, Khandeparker et al. 2002a, 2003), bryozoans (Kirchman & Mitchell 1983), polychaetes (Kirchman & Mitchell 1983, Qian 1999), oysters (Weiner et al. 1989), limpets (Zhao & Qian 2002), gastropods (Rodriguez et al. 1995), ascidians (Szewzyk et al. 1991) and hydroides (Leitz & Wagner 1993). In these studies, the inductive effect of biofilms on larval settlement is attributed to bacteria and their components in biofilms. In addition, many invertebrates show a gregarious response towards adult and juvenile conspecifics (e.g. barnacle cyprids or larvae of other species; Raimondi 1988). In the case of barnacles, arthropodin or settlement pheromone (a glycoprotein present in the adults; Knight-Jones & Crisp 1953) as well as its larval (cyprid) footprints (while exploring surfaces, cyprids leave behind footprints as temporary adhesives) have been reported to attract other barnacle larvae (Walker & Yule 1984, Clare et al. 1994). It has also been shown that specific small peptides can mimic barnacle pheromone and those with a basic carboxy-terminal amino acid and either a neutral or a basic amino-terminal amino acid enhance settlement and metamorphosis of the larvae of *Balanus amphitrite* (Tegtmeyer & Rittschof 1988). Browne & Zimmer (2001) reported that trace amounts of the synthetic peptide analog glycyl-glycyl-L-arginine (5×10^{-8} M), which is similar in structure to peptide signal molecules that are naturally released by the adult barnacles, significantly promoted the settlement of *B. amphitrite*. These studies indicate that subtle changes in seawater chemistry can influence habitat colonization. Recently, a thraustochytrid protist detected in marine microbial films has been shown to induce settlement of the barnacle *B. amphitrite* (Raghukumar et al. 2000). Surprisingly, few attempts have been made to elucidate invertebrate larval settlement cues from the

diatoms and their components, although these form a high proportion of biofilms, especially in illuminated areas.

Earlier studies on induction of settlement and metamorphosis in marine invertebrates by benthic diatoms mainly focused on invertebrate species with grazing juveniles, such as abalones (Kawamura & Kikuchi 1992, Slattery 1992), sea urchins (Tani & Ito 1979, Rahim et al. 2004) and sea cucumbers (Ito & Kitamura 1997). Until recently, very few studies have focused on larval settlement induction by benthic diatoms with respect to larval settlement behaviour of sedentary organisms such as the barnacle *Balanus balanoides* (Le Tourneux & Bourget 1988) and the polychaete *Hydroides elegans* (Harder et al. 2002, Lam et al. 2003). These studies focused mainly on the influence of biofilms dominated by benthic diatoms.

In the present study, efforts were made to investigate the influence of diatom films of different densities and their extracellular components on the larval metamorphosis of the barnacle *Balanus amphitrite*. The capabilities of 5 diatom species belonging to the genera *Navicula* and *Amphora*, both of which are dominant in microfouling and benthic microalgal communities, to induce larval metamorphosis of *Balanus amphitrite* were explored. The diatom species isolated from natural biofilms were used as candidate organisms. The effectiveness of different settlement-inducing components from these diatoms was assessed.

MATERIALS AND METHODS

Culturing conditions and source of algal material. Five species of pennate diatoms belonging to 2 genera—*Amphora* (*A. coffeaeformis* and *A. rostrata*) and *Navicula* (*N. cruciula*, *N. transitans* var. *derasa* f. *delicatula* and *N. subinflata*)—were used in this study. The cultures are maintained in the Marine Corrosion and Materials Research Division, National Institute of Oceanography, Goa, India. *A. rostrata*, *N. cruciula* and *N. subinflata* were originally isolated from the biofilm, whereas *A. coffeaeformis* and *N. transitans* were isolated from the intertidal sediments of Dona Paula Bay, west coast of India (15° 27' N, 73° 48' E). Cultures of these species were maintained in the exponential growth phase by weekly re-inoculation into fresh culture medium, prepared in autoclaved seawater (~35 psu) enriched with nutrients (f/2 media; Guillard & Ryther 1962). Cells were grown in Erlenmeyer flasks (100 ml) containing 50 ml of culture medium and were maintained at 27 ± 1°C under a light photocycle of 12:12 h light:dark.

Extraction of diatom EPS. Prior to the extraction of diatom EPS, diatom cultures were made axenic in order

to avoid the contribution of bacterial EPS. A 2-step method (including physical and chemical methods) was employed to obtain the axenic culture. First, the diatom cells were repeatedly sterilely washed (10 times) before and after mild sonication to remove loosely adhered bacterial cells. Thereafter, the diatom cells were incubated overnight in nutrient-enriched media (f/2) containing 1 mg ml^{-1} lysozyme to remove Gram-negative bacteria (Kim et al. 1999). The cultures were again repeatedly sterilely washed, and then checked microscopically for any cell wall permeabilization. After this, they were incubated in fresh sterile f/2 medium containing an antibiotic mixture (1 mg ml^{-1} of penicillin G, 0.5 mg ml^{-1} of streptomycin, and 0.2 mg ml^{-1} of chloramphenicol) for 24 h. The addition of antibiotics does not affect photosynthesis or C fixation by the algae (Goto et al. 1999, Wolfstein et al. 2002). The culture was then transferred to antibiotic-free medium and cultivated as above. Axenic culture was confirmed by the spread plate method using Zobell marine agar and fluorochrome staining with 4′6-diamidino-2-phenylindole (DAPI). This method of obtaining axenic culture was employed for all the diatom species. These axenic cultures were further used for EPS extraction and larval assays.

Mass cultures (1.5 l × 3 [replicates] = 4.5 l) of each species were raised in three 2 l Schott Duran bottles using f/2 medium, as described above. After attaining stationary phase (16 d), the supernatant was decanted to separate the cells attached to the culture flask. The decanted supernatant was pooled and used to isolate planktonic/free EPS whereas the attached cells were used for the isolation of biofilm EPS.

The biofilm EPS from the attached cells was extracted following the method described by Beech et al. (2000). The attached diatom cells were treated with isotonic Tris buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA) containing 2.5% NaCl and incubated overnight at 4°C. After incubation, the extracted biofilm EPS and supernatant were centrifuged separately at 6000 rpm (12 000 × *g*) at 4°C for 30 min followed by a 0.2 µm (Nucleopore) filtration to remove any cell material. The filtrate was concentrated in a stirred ultra centrifugation cell (Amicon) using an ultra filter with a molecular weight cut-off of 1000 Da. The carbohydrate content of the filtrate was quantified using the phenol-sulfuric acid method (Dubois et al. 1956), with glucose as a standard. At the time of harvesting EPS, after isotonic Tris-buffer treatment, cultures were pooled and checked microscopically for cell lysis. Simultaneously, cell counts were also made of the pooled cultures in order to express EPS production per cell. The same procedure was followed for all the diatom species. Isolation of EPS from non-axenic culture of *Navicula crucicula* and *N. subinflata*, which are abundant in the fouling community, was also carried out following a similar method to compare the results of axenic and non-axenic conditions. The EPS produced was further used for larval assays and Fourier-transformed infrared spectroscopy (FTIR) analysis.

The growth rates and generation times for all the diatom species used in the study were also calculated from the initial count of the inocula and final counts of the pooled cultures, which were made at the time of harvesting EPS. Counting of diatom cells was performed by light microscopy (Olympus BH2) using a haemocytometer.

Preparation of the adult extract (AE). Cypris larvae are discriminating in their choice of settlement site. Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics. This gregarious feature has been related to settlement pheromone, a glycoprotein present in the adults, also referred to as arthropodin (Knight-Jones & Crisp 1953). An extract was, therefore, prepared from the adult *Balanus amphitrite* and was considered as a positive control in the larval assays.

AE was prepared following the method described earlier by Larman et al. (1982). *Balanus amphitrite* adults, collected from the intertidal area of Dona Paula Bay (15° 27′ N, 73° 48′ E), were brought to the laboratory and cleaned by brushing off the epibiotic growth on their shells using a nylon brush. The barnacles were then washed and 100 g wet weight of whole adults were crushed with a mortar and pestle using 100 ml of deionised water (reverse osmosis pure). The supernatant of the crushed mixture was decanted, centrifuged at 12 000 × *g* for 5 min and thereafter boiled for 10 min in a boiling water bath. The extract was again centrifuged at 12 000 × *g* for 5 min and then frozen at –20°C until further use. The protein content of the extract was estimated following the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. A protein concentration of 50 mg µl^{-1} of AE was used for all assays (Rittschof et al. 1984).

Rearing of *Balanus amphitrite* larvae. *B. amphitrite* is a dominant fouling organism found all over the world and is the major target organism in biofouling studies as well as in the development of antifouling technology. *B. amphitrite*, an acorn barnacle, is an important model organism for these studies because of its rapid larval development, the ease of raising synchronous mass cultures and its predictable settlement under static conditions. This barnacle species is eurytolerant (Anil 1991, Anil et al. 1995), breeds throughout the year in waters around the subcontinent of India and is also dominant in the fouling community in Indian waters (Anil 1986). The life cycle of *B. amphitrite* includes planktotrophic larval development consisting of 6 naupliar instars and a non-feeding

cyprid instar. The first instar nauplii do not feed and molt into the second instar within 1 to 2 h. Instars II to VI are phytoplanktotrophic. Nauplii were mass reared in 2 l glass beakers using filtered seawater of 35 psu on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of 2×10^5 cells ml^{-1}. The food organism was replenished every day while changing the water. After 5 to 6 d, the cyprids obtained were siphoned out and stored at 5°C prior to settlement assays. Two d old cyprids were used to carry out the assays. These methods have been described in detail by Rittschof et al. (1984).

FTIR spectroscopy. Samples were prepared by grinding EPS with potassium bromide and pressing the mixture to form a pellet. FTIR spectroscopy (model Shimadzu 8201 PC) was used to read absorbance from 500 to 4000 cm^{-1} and detect the major structural groups of biofilm and free EPS produced by diatoms.

Assay protocol for Expts 1 and 2. Two different experiments were carried out, the schematic representations of which are given in Fig. 1. In Expt 1, the effectiveness of axenic and non-axenic diatom EPS both free and from biofilm was investigated. In Expt 2, the influences of diatom films (both axenic and non-axenic) at different cell densities were assessed. Along with these experiments, the effect of a positive control (AE) on larval metamorphosis was also investigated. The assay protocol employed is similar to that used for evaluating the influence of other microbial films and their components on barnacle larval settlement (Maki et al. 1988, Anil & Khandeparker 1998, Khandeparker et al. 2002a,b, 2003).

Expt 1. The settlement assays were carried out using 24-well multiwells (Corning-25820). The multiwells were inoculated with biofilm/free EPS from axenic/non-axenic diatom cultures. Controls were autoclaved seawater (ASW) (negative control) and AE (positive control). After 3 h, the inoculated multiwells were rinsed repeatedly with ASW under a laminar chamber. Subsequently, 1 cyprid was introduced with 2 ml of ASW (35 psu). The settlement assays were monitored for 4 d with an intermittent observation every day. Assay wells were maintained at 26 ± 1°C (12:12 h light:dark). The assays were conducted

separately with axenic and non-axenic diatoms, and were repeated 3 times by taking different batches of larvae (for each condition, n ≈ 10).

Expt 2. The settlement assays were carried out using 24-well multiwells (Corning-25820). The multiwells were inoculated aseptically under a laminar flow with axenic and non-axenic diatom cultures at densities of 200, 1000 and 5000 cells ml^{-1}. After inoculation, the multiwells were kept overnight for the formation of films. The transfer of larvae, number of replicates, monitoring of settlement assays and maintenance of assay wells were the same as described for Expt 1. As in Expt 1, the assays were conducted separately with axenic and non-axenic diatoms. The growth of diatoms in the multiwells was also assessed by using inverted
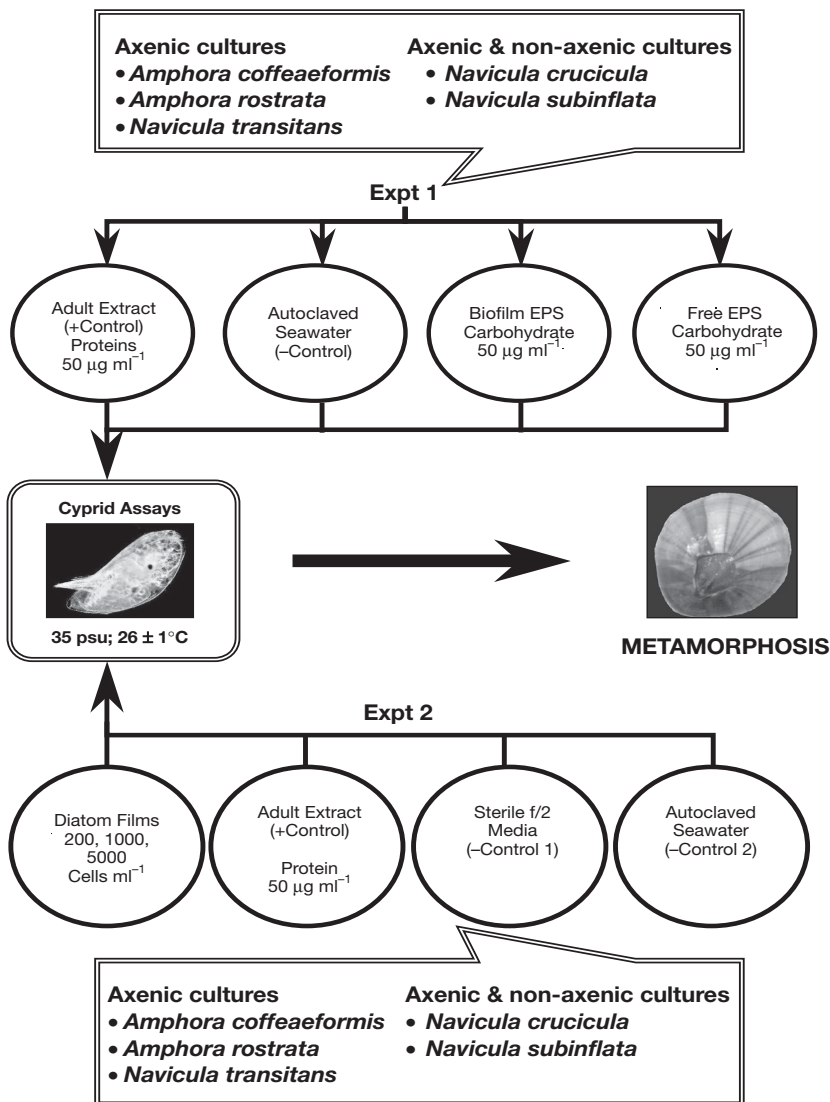

Fig. 1. Schematic representation of the experimental set-up. EPS: extracellular polymeric substances

microscopy along with the monitoring of settlement assays. In this experiment, an additional control, f/2 medium, was also used as a second negative control.

Data analysis. The data obtained are described as percentages of metamorphosed larvae in each assay condition. The data on metamorphosis (%) were arcsine-transformed before statistical analysis. To improve the transformation, a slight modification of the Freeman & Tukey (1950) transformation was used, namely, $p' = \frac{1}{2}\{[\arcsin\sqrt{X/(n + 1)}] + [\arcsin\sqrt{(X + 1)/(n + 1)}]\}$, where X is the number of larvae metamorphosed and n is the total number of larvae (Zar 1996). The normality of the data was checked with Shapiro-Wilks W-test (Shapiro & Wilk 1965). Data that failed to meet the homogeneity criterion were analyzed non-parametrically by transformation of values to ranks, followed by ANOVA and Tukey's multiple comparison test at the $\alpha = 0.05$ significance level (Conover & Iman 1981). These analyses were performed for each species (axenic and non-axenic), considering time as a variable separately to evaluate the differences between the treatments, i.e. control and EPS/films. In addition, 2-way ANOVA was carried out to evaluate the effect of diatom species (axenic and non-axenic) on larval metamorphosis. This analysis was performed for each of the treatments (biofilm and free EPS as well as diatom films of varying densities) separately, by considering species and time as variables, and the blank treatment was omitted from the analysis. Normality tests, ANOVA and Tukey's multiple comparison tests were carried out using the Statistica (StatSoft) software package.

RESULTS

Diatom growth and EPS production

Biofilm EPS production was comparatively higher than free EPS except for *Navicula transitans* (Fig. 2). *Amphora* spp., which have a faster growth rate and lesser generation time, produced higher biofilm EPS per cell compared to *Navicula* spp. (Figs. 2 & 3). Among the *Amphora* spp., *A. rostrata* produced higher EPS per cell followed by *A. coffeaeformis* (Fig. 2). In the case of *Navicula* spp., EPS production was in the following order: *N. crucicula* > *N. transitans* > *N. subinflata* (Fig. 2). However, *N. transitans* produced higher free EPS followed by *A. rostrata*, *A. coffeaeformis*, *N. subinflata* and *N. crucicula* (Fig. 2). In another set, it was found that the non-axenic culture of *N. subinflata* and *N. crucicula* produced higher biofilm EPS per cell than axenic cultures (Fig. 2). Results also revealed that the growth rate of non-axenic diatom

cultures was higher/faster than for axenic cultures (Fig. 3). This further indicates that the bacteria associated with the diatoms play an important role in the eco-biology of diatoms like growth rate and EPS production.
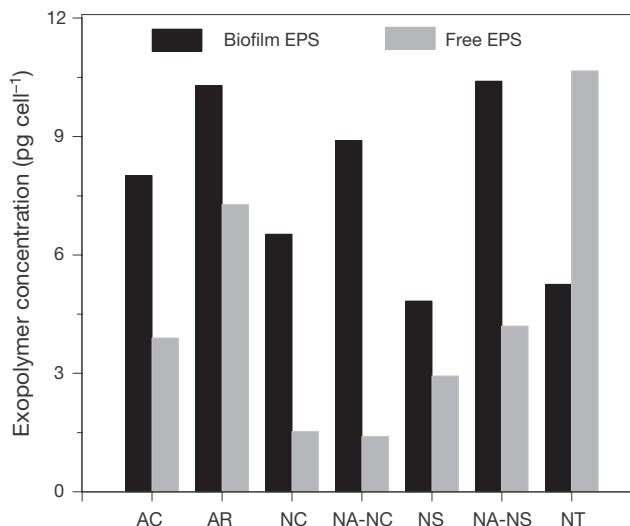

Fig. 2. Exopolymer production by diatoms under axenic and non-axenic conditions. AC: *Amphora coffeaeformis*; AR: *A. rostrata*; NC: *Navicula crucicula*; NT: *N. transitans*; NS: *N. subinflata*; NA-NC: non-axenic-*N. crucicula*; NA-NS: non-axenic-*N. subinflata*
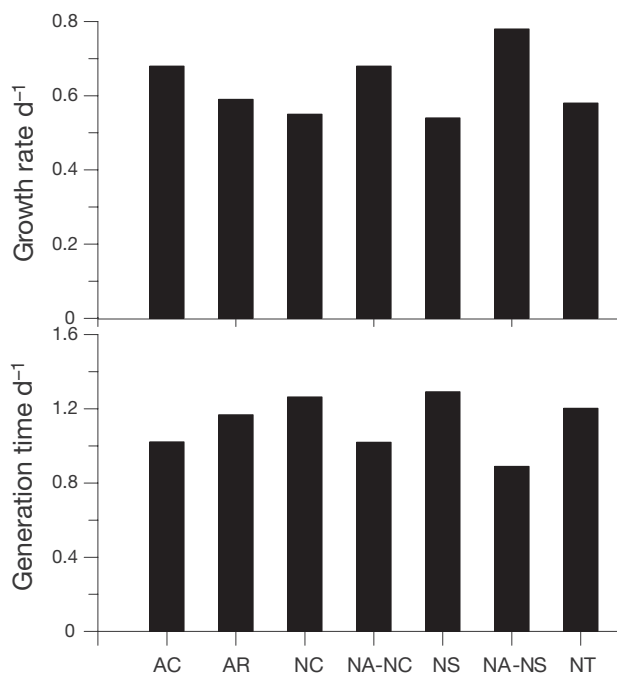

Fig. 3. Growth rate and generation time of diatoms grown under axenic and non-axenic conditions. AC: *Amphora coffeaeformis*; AR: *A. rostrata*; NC: *Navicula crucicula*; NT: *N. transitans*; NS: *N. subinflata*; NA-NC: non-axenic-*N. crucicula*; NA-NS: non-axenic-*N. subinflata*

Effects of diatom EPS on cyprid metamorphosis

The results of the larval assays revealed that the diatom EPS did not promote larval settlement induction compared to the positive control (AE) and the maximum percentage of larval metamorphosis was shown for biofilm EPS for all experimental days (Fig. 4). Tukey's multiple comparison test revealed

an insignificant variation between the biofilm EPS (axenic and non-axenic) and the positive control (AE) for all the species except for *Amphora rostrata*, for which biofilm EPS showed a weak inductive influence, and there was a significant difference with the negative control (FSW). The opposite results were found for free EPS (Table 1). This indicates that the biofilm EPS facilitated larval meta-
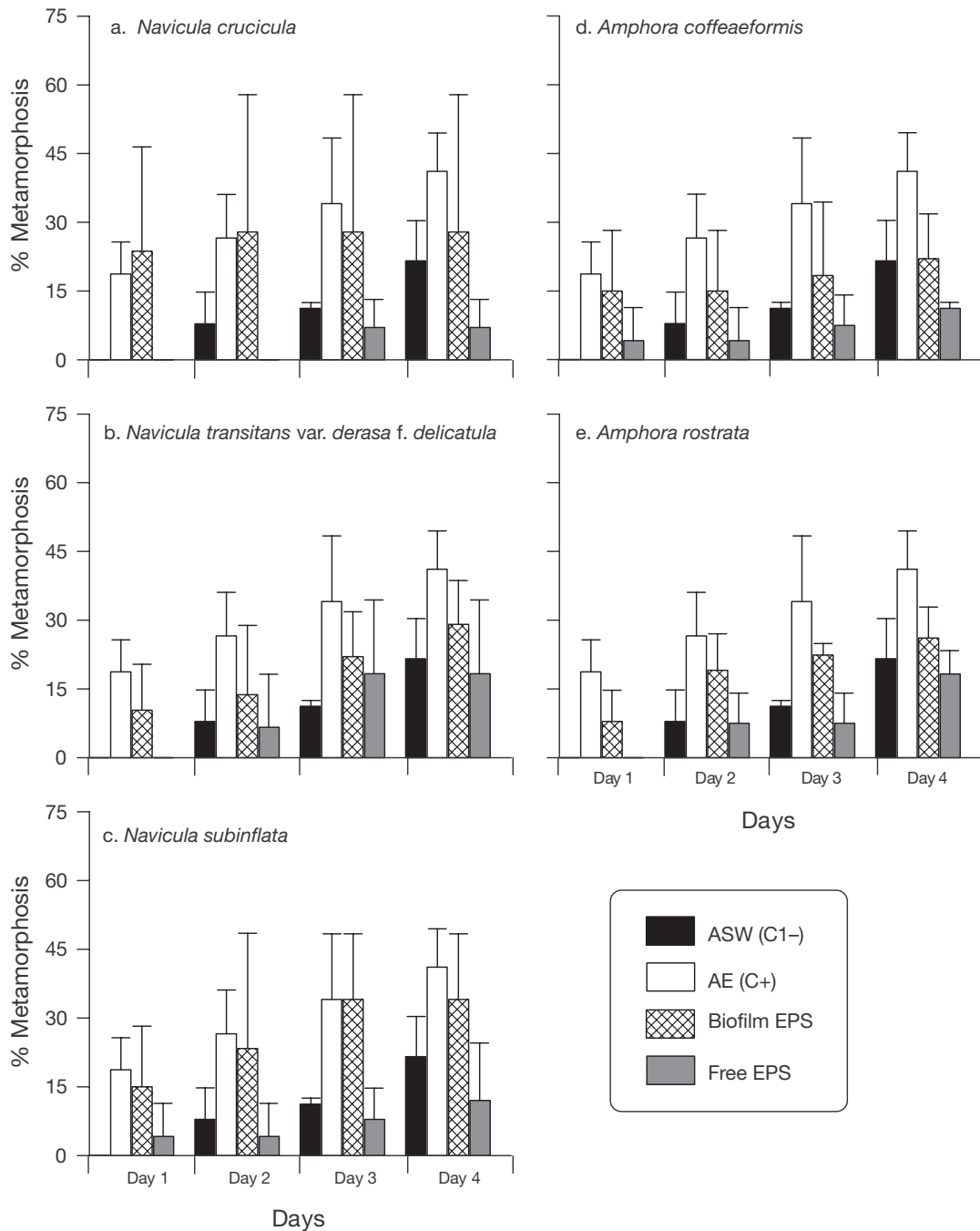

Fig. 4. *Balanus amphitrite*. Influence of diatom exopolymers produced under axenic conditions on the larval metamorphosis of *B. amphitrite*. ASW (C–): autoclaved seawater (negative control); AE (C+): adult extract (positive control)

Table 1. Tukey's multiple comparison test (α = 0.05; 2-way ANOVA, p < 0.001). Influence of biofilm and free extracellular polymeric substances (EPS) of *Navicula* spp. (*N. subinflata*, *N. crucicula* and *N. transitans*) and *Amphora* spp. (*A. coffeaeformis* and *A. rostrata*) obtained from different culture conditions (axenic and non-axenic) on the metamorphosis of *Balanus amphitrite* cyprids. Control (–): negative control (autoclaved seawater); Control (+): positive control (adult extract); NE: 'no effect' (nearly similar percentage of larval settlement as in the negative control); I: 'inductive' (same percentage of larval settlement as in the positive control); WI: 'weakly inductive' (percentage of larval settlement significantly higher and lower than in the negative and positive control, respectively); >: biofilm EPS showed maximum metamorphosis compared to that of free EPS; *: significant (p ≤ 0.05); ns: non-significant

| Species | Biofilm EPS | | Free EPS | | Biofilm vs. free EPS |
	Control (–)	Control (+)	Control (–)	Control (+)	
Axenic					
N. subinflata	*	ns (I)	NE (ns)	*	*(>)
N. crucicula	*	ns (I)	NE (ns)	*	*(>)
N. transitans	*	ns (I)	NE (ns)	*	ns
A. coffeaeformis	*	ns (I)	NE (ns)	*	*(>)
A. rostrata	*	*(WI)	NE (ns)	*	*(>)
Non-axenic					
N. subinflata	*	ns (I)	NE (ns)	*	*(>)
N. crucicula	*	ns (I)	NE (ns)	*	*(>)

morphosis, further indicating that the signals vary depending upon the species (Table 1). In contrast, free EPS showed no effect on larval metamorphosis (Table 1). Among the diatom genera, EPS of *Navicula* spp. showed higher rates of larval metamorphosis compared to *Amphora* spp. (Fig. 4). However, 2-way ANOVA showed insignificant variations between the species (for both inter-generic and intra-generic) (Table 2a). Among the non-axenic cultures, the biofilm EPS of *N. crucicula* showed higher metamorphosis compared to AE, but this was not the case for *N. subinflata* (Fig. 5). Two-way ANOVA also showed significant variation between the species (Table 2b). Although the biofilm EPS of both axenic and non-axenic cultures of *N. crucicula* and *N. subinflata* facilitated larval metamorphosis, among them, biofilm EPS of non-axenic cultures promoted larval metamorphosis better than axenic cultures with respect to the positive control (Figs. 4 & 5).

Table 2. Two-way ANOVA. Influence of biofilm and free EPS of different diatom species, obtained from different culture conditions (axenic and non-axenic), on the metamorphosis of *Balanus amphitrite* cyprids

| Effect | df | Biofilm EPS | | | Free EPS | | |
		MS	*F*	p	MS	*F*	p
(a) Axenic							
Species	4	190.65	0.57	0.684	192.24	0.68	0.612
Days	3	892.63	2.68	0.060	1481.74	5.22	0.004
Combined	12	82.74	0.25	0.994	91.29	0.32	0.981
(b) Non-axenic							
Species	1	155.04	15.42	0.001	70.04	4.31	0.054
Days	3	251.92	25.06	<0.001	239.25	14.71	<0.001
Combined	3	8.79	0.87	0.475	13.68	0.84	0.491

Effects of diatom films on cyprid metamorphosis

The results of larval assays are shown in Fig. 6. In the cases of *Navicula transitans*, *N. subinflata* and *Amphora coffeaeformis*, induction of metamorphosis was better than the positive control at higher cell densities, whereas for *N. crucicula* and *A. rostrata*, this was seen at lower cell densities (Fig. 6). Two-way ANOVA showed significant variation between the treatments, i.e. diatom films of varying densities and controls (ANOVA: n = 72, p < 0.001) for each species. For *N. transitans* and *A. coffeaeformis* (1000 and 5000 cells ml^{-1}), and *N. subinflata* (1000 cells ml^{-1}), Tukey's multiple comparison test revealed insignificant variation with respect to the positive control and significant variation with respect to the negative control (Table 3). This indicates that these diatoms facilitated larval metamorphosis at higher cell densities. The promotion of metamorphosis by the diatom films at higher cell densities was in the following order: *N. transitans* > *A. coffeaeformis* > *N. subinflata* (Table 3, Fig. 6). In the case of *N. crucicula* and *A. rostrata*, larval metamorphosis was facilitated at lower cell densities (Table 3). Two-way ANOVA also showed significant variation between the species and days at all cell densities (Table 4a). The percentage of larval metamorphosis in all treatments showed a significant increment from Days 1 to 4 (ANOVA: n = 72, p < 0.001). For *A. coffeaeformis*, on Day 4, metamorphosis increased with an increase in cell density, whereas the reverse was observed for *N. cruci-*
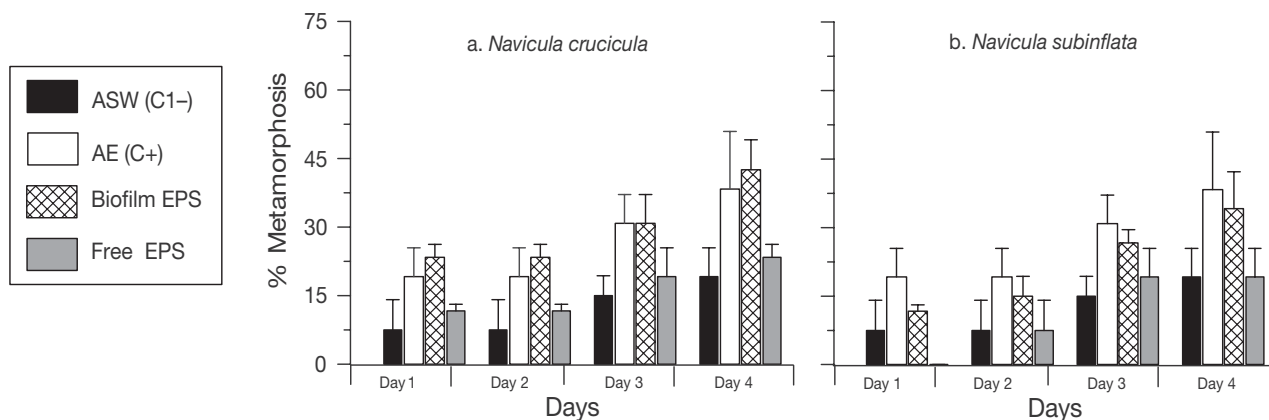
Fig. 5. *Balanus amphitrite*. Influence of diatom exopolymers produced under non-axenic conditions on the larval metamorphosis of *B. amphitrite*. ASW (C–): autoclaved seawater (negative control); AE (C+): adult extract (positive control)

cula and *A. rostrata*. These results suggest that larval metamorphosis is species- and density-dependent. Assays with films of non-axenic diatoms revealed that the larval metamorphosis increased with cell density (Table 3, Fig. 7). In contrast to films of axenic diatoms, films of non-axenic diatoms promoted larval metamorphosis, i.e. the percentage of metamorphosis exceeded that of the positive control (Table 3, Figs. 6 & 7). Growth of diatoms in the assay wells was also observed during the experiment for both axenic (Fig. 8) and non-axenic (Fig. 9) diatoms. Under axenic conditions, the growth rate of *Amphora* spp. was higher at all densities compared to that of *Navicula* spp. (Fig. 8); growth rates of *N. crucicula* and *N. subinflata* were higher under non-axenic conditions (Figs. 8 & 9).

FTIR analysis

The FTIR spectra of biofilm and free EPS of the diatoms chosen for the study showed similar characteristic strong peaks around 3429 to 3367, 1629 to 1612, 1460 to 1406, 1271 to 1259 and 1122 to 1039 cm^{-1} for O-H stretching, N-H bend, C-H bend, sulfate and C-O stretching, respectively (data not shown). The FTIR results reveal that the primary structural components of diatom EPS (both biofilm and free) are carbohydrates. However, the N-H bend indicates the presence of proteins.

DISCUSSION

Compared to positive and negative controls, the results obtained for the larval assays categorized the settlement responses into 3 distinct groups: (1) 'induc-

tive': percentage of metamorphosis is greater or equal to positive control; (2) 'weakly inductive': percentage of metamorphosis is greater than in the negative control but less than in the positive control; and (3) 'no effect': percentage of metamorphosis is equal to or less than in the negative control. This classification is similar to that by Harder et al. (2002) for the larvae of the fouling polychaete *Hydroides elegans*. The results revealed that the biofilm EPS produced by the diatoms represented Category 1, except *A. rostrata* that fell into Category 2, whereas free EPS represented Category 3, with an inductive effect on larval settlement of *Balanus amphitrite* (Table 1). Two-way ANOVA showed significant variation between the treatments (controls and EPS) and days (ANOVA: n = 48, p < 0.001). This indicates that the diatom EPS strongly promoted larval metamorphosis like in several bacterial strains (Anil & Khandeparkar 1998, Khandeparkar et al. 2002a). Induction of larval metamorphosis by bacteria has been attributed to bacterial secondary metabolites and glycoproteinacious bacterial exopolymers (Unabia & Hadfield 1999). Similar conclusions were drawn with respect to the induction of larval settlement by natural biofilms in other invertebrate taxa (Johnson et al. 1991, Szewzyk et al. 1991, Leitz & Wagner 1993). This indicates that biofilm comprising much diatom EPS may give weak to strong positive cues for the incoming larvae depending on the species. Among the axenic diatoms, although *Amphora* spp. yielded more biofilm EPS per cell, they were less influential on larval metamorphosis than *Navicula* spp. (Figs. 2, 4 & 5). However, ANOVA showed insignificant variation between the species (Table 2a). Biofilm EPS of non-axenic *N. crucicula* and *N. subinflata* represented Category 1, whereas free EPS represented Category 3 (Table 1). This indicates that biofilm EPS produced by axenic and non-axenic diatoms facilitated larval metamorphosis,
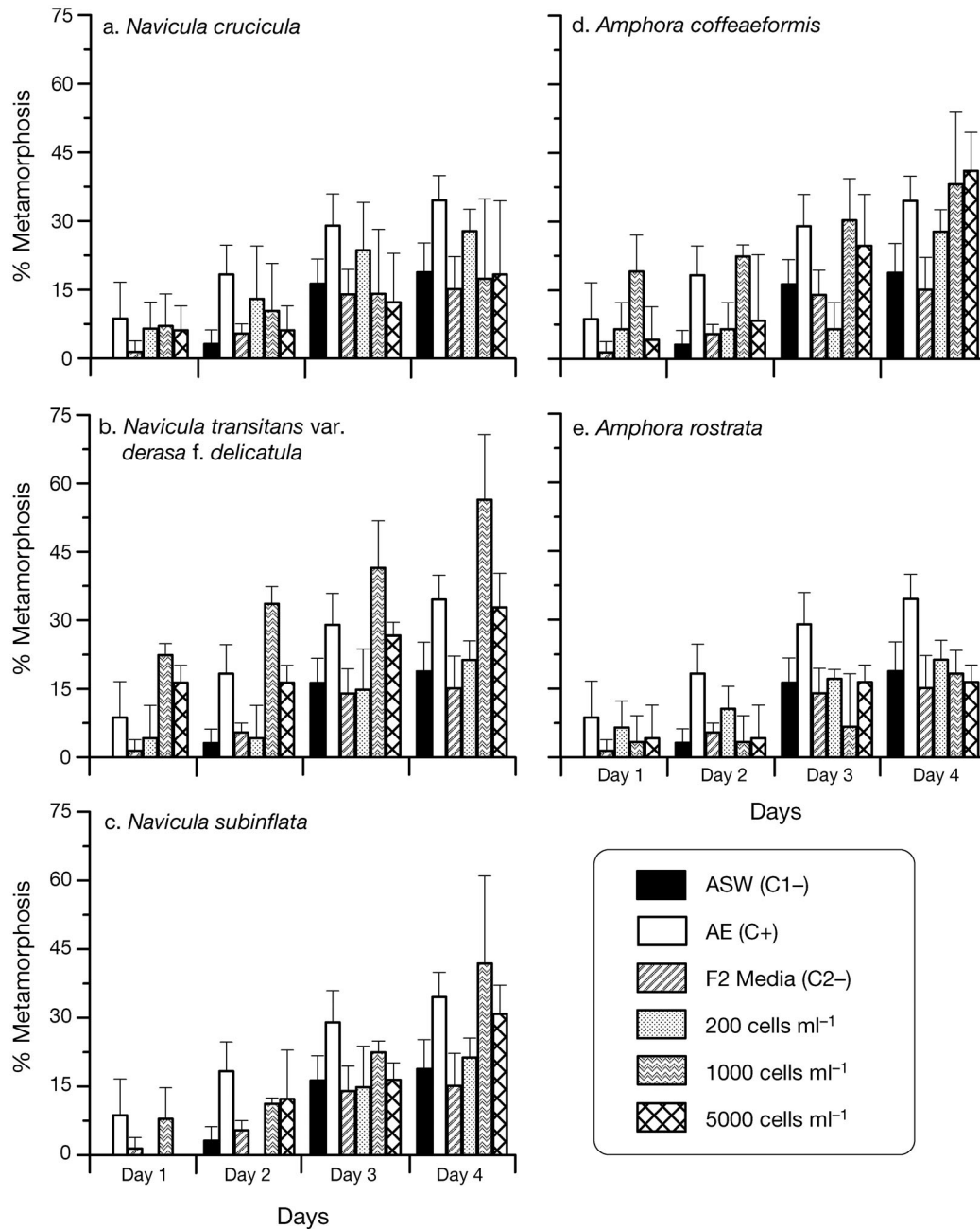
Fig. 6. *Balanus amphitrite*. Influence of axenic diatom films at 3 different densities on the larval metamorphosis of *B. amphitrite*. ASW (C−): autoclaved seawater (negative control); AE (C+): adult extract (positive control)

although it was observed that the average percentage larval metamorphosis in the latter was higher (Figs. 4a,c & 5). Significant species-specific variation of non-axenic diatoms was also observed with respect to larval metamorphosis (Table 2b, Fig. 5), possibly because of differences in the quality of EPS. Grossart (1999) reported that bacteria and diatoms not only grew well together in f/2 medium or artificial seawater but also that the amount and quality of exopolymeric matter of the mixed and pure cultures differed. This further suggests that the diatoms in association with bacteria not only influenced the enhanced production of EPS (Fig. 2) but also mediated larval metamorphosis.

Reports in the literature suggest that the percentage composition of monosaccharides in diatom EPS differs between species (Table 5). A summary of the monosaccharide composition of some diatoms selected in the study and based on the available literature is shown in

Table 3. Tukey's multiple comparison test (α = 0.05; 2-way ANOVA, p < 0.001). Influence of diatom films of different densities of *Navicula* spp. (*N. subinflata*, *N. crucicula*, *N. transitans*) and *Amphora* spp. (*A. coffeaeformis* and *A. rostrata*) grown under varying culture conditions (axenic and non-axenic) on the metamorphosis of *Balanus amphitrite* cyprids. C (+): positive control (adult extract); C1 (–): negative control 1 (autoclaved seawater); C2 (–): negative control 2 (f/2 media); NE: 'no effect' (same percentage of larval settlement as in the negative control); I: 'inductive' (nearly similar percentage of larval settlement as in the positive control); >: maximum metamorphosis at higher densities; <: minimum metamorphosis at lesser densities; *: significant (p $\leq$ 0.05); ns: non-significant

| Species | 200 cells ml^{-1} | | | 1000 cells ml^{-1} | | | 5000 cells ml^{-1} | | | Diatom films (cells ml^{-1}) | | |
	C (+)	C1 (–)	C2 (–)	C (+)	C1 (–)	C2 (–)	C (+)	C1 (–)	C2 (–)	200 vs. 1000	200 vs. 5000	1000 vs. 5000
Axenic												
N. subinflata	*	ns (NE)	ns (NE)	ns (I)	*	*	*	ns (NE)	ns (NE)	*(<)	ns	ns
N. crucicula	ns (I)	*	ns (NE)	ns (I)	ns (NE)	ns (NE)	*	ns (NE)	ns (NE)	ns	ns	ns
N. transitans	*	ns (NE)	ns (NE)	*(I)	*	*	ns (I)	*	*	*(<)	*(<)	*(>)
A. coffeaeformis	*	ns (NE)	ns (NE)	ns (I)	*	*	ns (I)	ns (NE)	ns (NE)	*(<)	ns	*(>)
A. rostrata	ns (I)	ns (NE)	ns (NE)	*	ns (NE)	ns (NE)	*	ns (NE)	ns (NE)	ns	ns	ns
Non-axenic												
N. subinflata	*	ns (NE)	ns (NE)	ns (I)	*	*	ns (I)	*	*	*(<)	*(<)	ns
N. crucicula	*	ns (NE)	ns (NE)	ns (I)	*	*	ns (I)	*	*	*(<)	*(<)	ns

Table 4. Two-way ANOVA. Influence of monospecific films of different diatom species grown under different conditions (axenic and non-axenic) at different cell densities (200, 1000 and 5000 cells ml^{-1}) on the metamorphosis of *Balanus amphitrite* cyprids

| Effect | df | 200 cells ml^{-1} | | | 1000 cells ml^{-1} | | | 5000 cells ml^{-1} | | |
		MS	*F*	p	MS	*F*	p	MS	*F*	p
(a) Axenic										
Species	4	402.57	3.44	0.017	2148.34	18.91	<0.001	720.13	5.97	0.001
Days	3	3354.63	28.66	<0.001	1311.54	11.55	<0.001	2894.08	23.98	<0.001
Combined	12	120.01	1.03	0.446	62.93	0.55	0.865	119.41	0.99	0.475
(b) Non-axenic										
Species	1	26.04	0.96	0.343	54.00	1.51	0.237	0.67	0.04	0.837
Days	3	206.42	7.57	0.002	130.56	3.65	0.035	275.44	18.04	<0.001
Combined	3	6.85	0.25	0.859	18.11	0.51	0.684	11.22	0.73	0.546
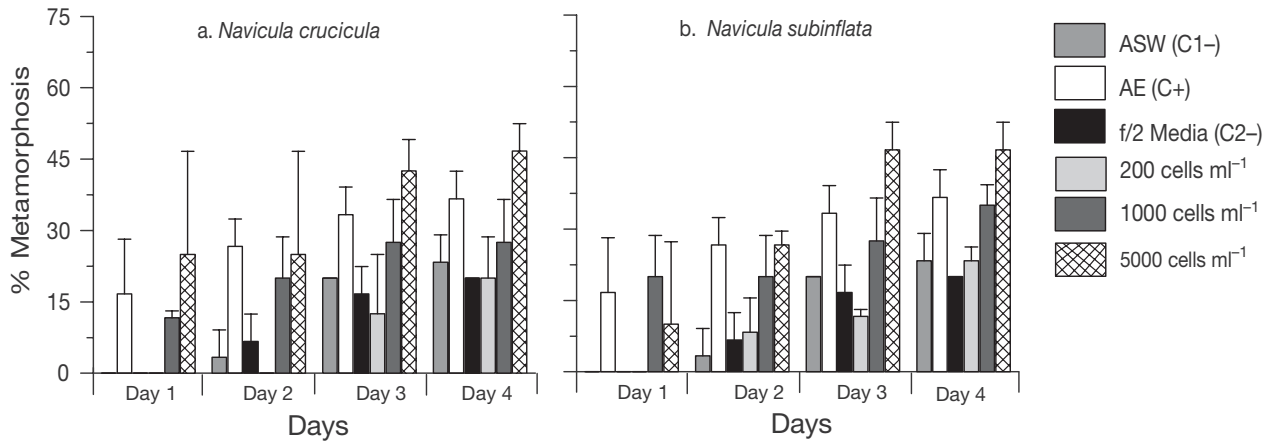
Fig. 7. *Balanus amphitrite*. Influence of non-axenic diatom films at 3 different densities on the larval metamorphosis of *B. amphitrite*. ASW (C–): autoclaved seawater (negative control); AE (C+): adult extract (positive control)
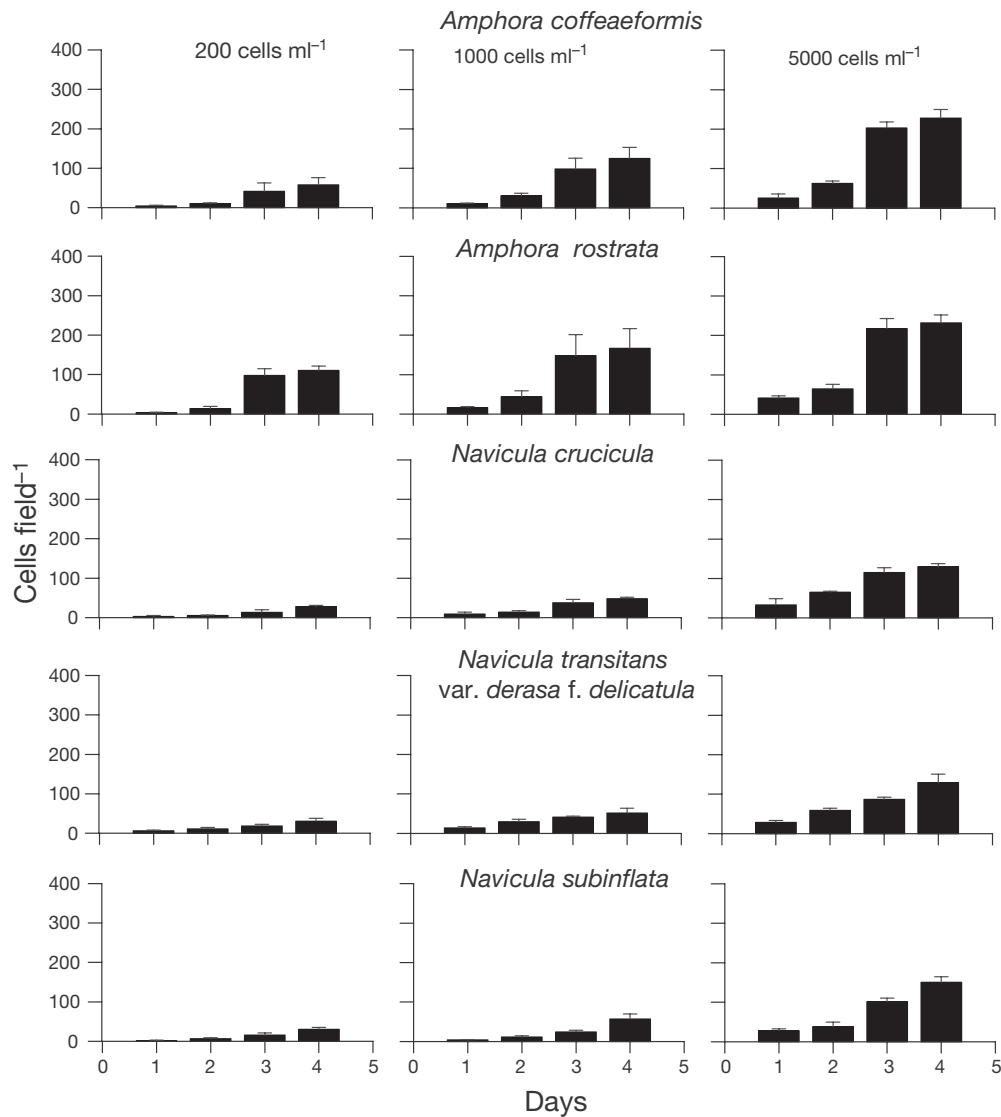
Fig. 8. Growth of diatoms observed in the assay wells during the larval assays with axenic diatom films at 3 different densities

Table 5; data obtained from Bhosle et al. (1995, 1996) and Khandeparker & Bhosle (2001) are from the same species used in this study. The monosaccharide composition of the diatom EPS revealed that glucose is the most dominant monosaccharide in the EPS produced by *Navicula subinflata*, whereas for *Amphora* spp., glucose, galactose and xylose are dominant (Table 5).

Neal & Yule (1996) consider that the structure of bacterial EPS is capable of either determining the effectiveness of the cyprid's temporary adhesive or of affecting the cyprid's willingness to detach (Yule & Walker 1984). This indicates that the difference in the chemical composition of the EPS produced by different species of benthic diatoms in the biofilm may influence the effectiveness of cyprid metamorphosis. FTIR spectra of the diatom EPS appear similar for all the diatom spe-

cies, i.e. they did not reveal inter- or intra-generic variation (data not shown). This indicates that EPS produced by diatoms are of similar nature, mainly comprising carbohydrates with proteins. However, the percentage composition varies according to species (Table 5), which might have influenced the rate of metamorphosis among the diatom species. Earlier observations have hypothesized that sugars in solution adsorb electrostatically through −OH groups to polar groups associated with the cyprid larvae's temporary adhesive (CTA) (Yule & Walker 1987). It has been suggested that *Lens culinaris* agglutinin (LCA-binding sugar chains (i.e. D-glucose and D-mannose) of the AE are involved in the settlement of *Balanus amphitrite* (Matsumura et al. 1998). Khandeparker et al. (2002b) reported that the D-mannose sugar-treated cyprids
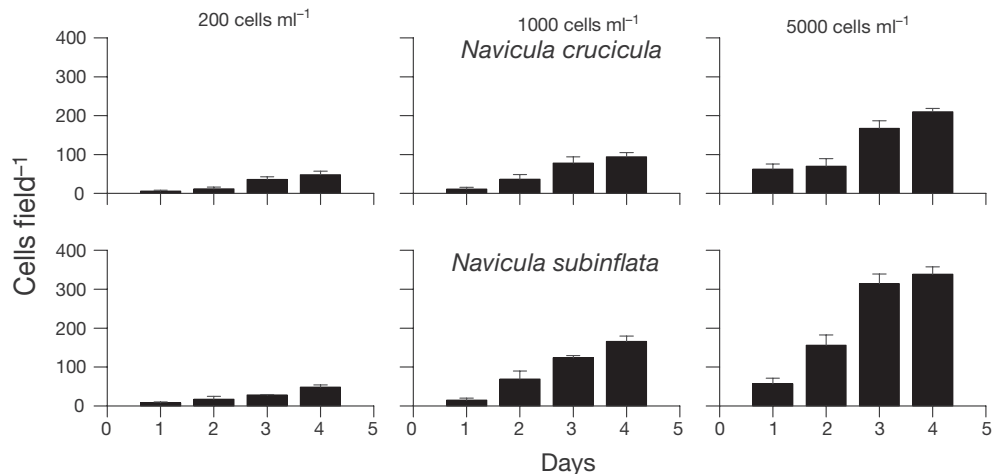
Fig. 9. Growth of diatoms observed in the assay wells during the larval assays with non-axenic diatom films at 3 different densities

Table 5. Summary of the monosaccharide composition (relative %) of the diatom EPS based on the literature

Species	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
Amphora coffeaeformis[a]							
Biofilm EPS							
Mechanically isolated capsules	9.0	9.0	–	9.0	13.0	13.0	40.0
Water soluble material	8.0	31.0	2.0	8.0	3.0	26.0	20.0
Water insoluble material	13.0	32.0	–	13.0	5.0	32.0	5.0
Amphora coffeaeformis[b]							
Capsular EPS	0.5	–	–	–	11.9	4.8	81.8
Amphora rostrata[c]							
Biofilm EPS	7.4	36.7	8.7	2.1	8.7	27.6	8.7
Free EPS	7.6	41.0	4.3	2.3	9.1	32.0	3.3
Navicula subinflata[d]							
Biofilm EPS	0.5	0.4	0.2	0.8	1.7	2.2	94.0

[a]Wustman et al. (1997); [b]Bhosle et al. (1996); [c]Khandeparker & Bhosle (2001); [d]Bhosle et al. (1995)

triggered metamorphosis but that the LCA-specific sugars D-glucose and D-galactose had a smaller effect. Based on these earlier reports, it is possible to infer that the qualitative and quantitative variations in the exopolymers produced by the diatoms can yield different cues.

The experiments with diatom films at different densities revealed that the larval metamorphosis is species- and density-dependent (Tables 3 & 4, Figs. 6 & 7). Diatom films at higher cell densities — *Navicula transitans* and *Amphora coffeaeformis* (1000 and 5000 cells ml^{-1}, respectively), and *N. subinflata* (1000 cells ml^{-1}) — inoculated in the assay wells represented Category 1, whereas diatom films of lower cell densities (200 cells ml^{-1}) represented Category 3, with reference to the inductive effect on larval settlement of *Balanus amphitrite* (Table 3). This indicates that these species, which are dominant in the natural biofilm (Mitbavkar

& Anil 2002, authors' pers. obs.), can play an important role in the population dynamics of *B. amphitrite* by influencing the induction of larval settlement. *A. rostrata* and *N. crucicula* at lower cell densities (200 cells ml^{-1}) represented Category 1 with an inductive effect on larval settlement of *B. amphitrite* (Table 3). In non-axenic cultures of *N. crucicula* and *N. subinflata*, the wells inoculated with 1000 and 5000 cells ml^{-1} represented Category 1, whereas wells inoculated with 200 cells ml^{-1} represented Category 3 (Table 3, Fig. 7). This further indicates that larval metamorphosis increased with an increase in cell density (Fig. 7). Lam et al. (2003) also reported a clear density-dependent effect of diatom surface coverage on the induction of polycheate *Hydroides elegans* larval settlement. Our study and earlier studies (Lam et al. 2003) suggest that the diatom density/coverage deserves consideration regarding its importance in mediating invertebrate lar-

Table 6. Size, shape and nature of the film observed during investigation for the diatom used in the study. L: length; B: width

Species	Size (μ)		Shape	Film type
	L	B		
Amphora coffeaeformis	23	9	Cymbelloid	Aggregate
Amphora rostrata	57	17	Cymbelloid	Uniform
Navicula crucicula	10	3	Prism on elliptical base	Uniform
Navicula transitans	40	11	Prism on elliptical base	Aggregate
Navicula subinflata	17	3	Prism on elliptical base	Aggregate

val metamorphosis. However, in comparison with axenic films, non-axenic films promoted larval metamorphosis (Table 3, Figs. 6a,c & 8), indicating the influence of surface-associated bacteria in combination with diatoms on larval induction.

The difference in growth rate observed for different species under axenic and non-axenic conditions (Figs. 3, 8 & 9) might have resulted in microscale variations on the surface of the assay wells. The diatoms used in the study had varying cell size, shape and behavior (aggregation and spreading) (Table 6). *Navicula transitans*, *N. subinflata* and *Amphora coffeaeformis* initially formed the film by spreading uniformly and thereafter aggregated into small colonies, whereas *A. rostrata* (largest in size) and *N. crucicula* (smallest in size) developed a uniform film. The induction of larval metamorphosis was higher for the former 3 species (Fig. 6). Hence, the observed microscale variations in the growth pattern exhibited by an individual species population might play an important role in the population dynamics of *Balanus amphitrite*. Qian et al. (2003) hypothesized that changes in the microbial community lead to changes in the recruitment pattern of marine invertebrates at a microscopic scale.

In conclusion, larval assays revealed that the benthic diatom films and biofilm EPS of axenic diatoms facilitated larval metamorphosis, whereas free EPS offered no signals. In the case of non-axenic diatoms, films and their biofilm components also promoted larval metamorphosis. However, the cues varied from weak to strong depending upon the species and their density in the films, as well as the composition of EPS produced. This investigation indicates that the bacteria associated with diatoms in association with diatoms play an important role not only in enhancing in EPS production but also in providing cues to invertebrate larvae.

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