Cell–cell and cell–surface interactions in an illuminated biofilm: Implications for marine sediment stabilization†

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Article

Most wetted surfaces that are illuminated support a population of phototrophs. The marine sediment is no exception and there the major component of the microphytobenthic population is diatoms. These organisms are credited with stabilizing the sediment against physical disturbance by virtue of the extracellular carbohydrate polymers that they elaborate. However, diatoms synthesize and secrete several carbohydrate polymers and it is not certain which of them is involved in the stabilization process. In order to investigate this, we have constructed small glass bead-filled flow through bioreactors to mimic marine sediments. The flow rate through the bioreactors was found to reflect the physical stability of the bead bed. Thus flow rate was measured as a function of diatom growth and the production of three operationally-defined polymers, *i.e.*, those soluble in the medium, those soluble in 0.5 M $NaHCO_3$ at 90 °C and those not soluble in either solvent (matrix polymer). Growth of the diatoms did not change the hydraulic conductivity of the bioreactors. For *Amphora coffeaeformis*, neither did the production of medium-soluble nor $NaHCO_3$-soluble polymers. However, matrix polymer accumulation was directly correlated with a reduction in flow (regression coefficient $R^2 = 0.96$) and stabilization against physical disturbance. Results with species of *Navicula* were not as clear. Both $NaHCO_3$-soluble and matrix polymers were involved in producing the flow reduction. In the same manner we also measured the effect of *Pseudoalteromonas haloplanktis* growth on bead bed hydraulic conductivity and bead bed stability. Growing alone, no effect was found, but in co-culture with a single diatom species, the bacteria reduced the diatom effect on flow through the bioreactors seen earlier, however did not reduce the extent of their growth. Confocal scanning laser microscopy of beads colonized with diatoms alone, or diatoms in co-culture with bacteria, revealed that *P. haloplanktis* was able to inhibit diatom adhesion to the beads. When the bacteria were present there was less matrix polymer evident. We speculate that this interference with diatom metabolic activity was either the result of less matrix polymer synthesis, or its hydrolysis by the bacteria. The results are applicable to mixed species biofilms of this type on surfaces other than sediments.

1. Introduction

Intertidal mudflats form an important part of an estuarine ecosystem.[1,2] Their position between the land and the open ocean is responsible for the fact that they bear much of the impact of human activities on the aquatic environment. It is well recognized that the microphytobenthic organisms inhabiting intertidal sediments are responsible for a large part of estuarine productivity[3,4] and also that of sandy beaches.[5,6] Related to this is their involvement in the stabilization of estuarine sediment particles against physical disturbance. There is a large body of literature on the physical stabilization of marine sediments[3,7–18] and most investigators agree that it is the activities of the microphytobenthic organisms, most likely those of the diatoms, that play a major role in the prevention of sediment movement by wave action. The diversity of microorganisms in a nearshore environment is very large but we are not certain which organisms are most active in the stabilization process. Although Rao and Lewin[19] found 352 species of diatoms in the sediments of a small bay with stabilized areas, it is usually members of the genera *Amphora, Navicula* and *Nitzschia* that are most often seen in those areas.[10] It is not possible to exclude the activities of the benthic bacteria from this process,[20] but from a study of the correlation of the chlorophyll *a* content of sediments and their bacterial density, Underwood and Paterson[21] concluded that bacteria did not contribute appreciably to sediment stability. Van Duyl *et al.*[15] studied the coupling between carbohydrate in sediments and the activity of the indigenous bacteria. Their results suggest that a net increase in water-extractable carbohydrate attributable to diatom activity was indirectly coupled to bacterial productivity. Similarly, Murray *et al.*[23] showed that the incorporation of ^{3}H-thymidine into heterotrophic bacterial DNA was coupled to the phototrophic activity of diatoms in a mixed bacterial/diatom biofilm.

The exact manner in which diatoms contribute to the stabilization process is still not entirely agreed upon. There is a consensus however that the extracellular polymers produced by these organisms are responsible (*e.g.*, ref. 3), but it is uncertain which of the several polymers produced by diatoms has the major role. For instance, in most papers where natural (as opposed to model) sediments were sampled, there was a variable correlation between the chlorophyll *a* extracted from the sediment (a biomass indicator) and the amount of colloidal carbohydrate extracted (putative sediment stabilization indicator),[3] depending whether the extracts were made from ridges or runnels of a mudflat area. There were no correlations when the samples were taken from a sandy area of the beach. Underwood and Paterson did find a correlation between the extractable colloidal carbohydrate of sediments and their chlorophyll *a* content, but there was no correlation between the EDTA-extractable capsular carbohydrates from the same

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sediments and the erosion threshold for the sediment particles. De Winder *et al.*[5] had very similar results to Underwood and Paterson,[21] but Riethmuller *et al.*[22] found that the chlorophyll *a* content of sediments was not correlated with their erodibility and that the results obtained were site specific.

There are possibly several reasons for the differing results obtained by the various groups of workers. Of primary importance is the one mentioned by Dade *et al.*[20] who cautioned that it is not the mere presence of organisms, but their metabolic activities that is driving the stabilization process. This factor is not always considered. Secondarily, the processing of samples is not uniform between laboratories. Whereas some investigators[3,5] extracted sediments that had been lyophilized, others worked with fresh[22] or frozen[21] sediments. Lastly Decho[13] has observed that EPS is merely an operationally defined substance. It is not a specific compound with defined properties and its ability to be extracted with a particular solvent will vary between organisms and with their physiological state. Further, Decho[13] has observed that it will be important to examine the cohesive properties and viscosities of different types of diatom EPS to understand why some mats may or may not exhibit stabilizing effects on sediment. We have started to address this idea.

In view of the variability of natural sediments and perceived problems with sampling that are related to environmental patchiness, we have designed a completely artificial model system in order to answer questions related to the roles of bacteria and diatoms in sediment stabilization. The work is relevant to the physiological interaction of these organisms in any illuminated biofilm whether it be on the surface of a marine sediment, or on a marine structure such as a ship.

2. Materials and methods

2.1 Growth media

Artificial seawater medium modified to contain 5 mM calcium was used for diatoms (ASP_2,[25]) and marine broth, 2216[Difco] for bacteria. For some experiments bacteria and diatoms were grown in ASP_2 enriched with 0.2% D-glucose and 0.05% yeast extract [HO medium]. Mixotrophic (10–15 $\mu mol\ m^{-2}\ s^{-1}$) and heterotrophic growth (darkness) experiments with diatoms were carried out with the addition of 0.5 mM organic substrates, as described previously.[24]

2.2 Organisms

The diatoms were isolated from stabilized patches of sediment. *Amphora coffeaeformis* was isolated from a mangrove swamp drainage[24] and *Navicula* sp.1 was isolated from False Bay, San Juan Is., WA. Axenic cultures were made by picking colonies from streaked plates and re-streaking the enrichments until there was no growth of contaminating bacteria in ASP_2 seawater medium enriched with 0.05% D-glucose and 0.02% yeast extract. Bacteria were isolated from the same sediment sample as the diatom *Navicula* sp.1. The sediment particles were washed aseptically with marine medium by decantation. The washed particles were then treated with ultrasound in a low power sonic cleaning bath to remove bacteria attached to sediment particles. This enrichment of attached bacteria was used to inoculate 2216 petri plates for the isolation of experimental organisms. The bacterium used here was identified by its 16-S RNA sequence and fatty acid methyl ester profile as *Pseudoalteromonas haloplanktis* by MIDI labs, Newark, DE and Microcheck Inc., Northfield Falls, VT.

Diatom inocula for experimental purposes were grown at 25 °C and 80–100 $\mu mol\ m^{-2}\ s^{-1}$ without shaking until mid logarithmic phase (3–400 000 cells mL^{-1}). In some instances, diatoms grown for extraction of polymers were kept in 2.8 L Fernbach flasks containing 250 mL medium. Polymers were extracted from both fresh and lyophilized cells.

Bacteria were grown until late logarithmic phase (absorbance at 660 nm = 0.5–0.7) with shaking (200 rpm) in 15 mL medium contained in a 125 mL flask with four baffles.

2.3 Analytical methods

Carbohydrates in solution and attached to glass beads were determined by the phenol/sulfuric acid method of Dubois *et al.*[26] and orthophosphate by that in ref. 27. Chlorophyll was determined fluorimetrically.[28] Diatom cells were counted in a hemocytometer. At least 400 cells were counted for each determination to achieve a coefficient of variation (CV) of 10%. Protein was determined by the method of Lowry *et al.*[29] Results are presented as means $\pm$ standard deviations. Where these are not shown on graphs it is because they are less than the size of the data point.

2.4 Model sediments

Model sediments in the form of columns of glass beads contained in 10 mL disposable syringes were used as the experimental device. The rationale for this approach was that as the interstitial spaces in the bead bed were occluded by polymers, the flow rate for the column should become reduced. Several methods to produce these bioreactors were investigated. Methods wherein the beads were sterilized *in situ*, wet or dry, proved to be unsatisfactory in that the flow rates of columns were not reproducible. Pre-sterilized columns filled aseptically with a sterilized suspension of beads also gave unacceptable flow rates because the numbers of very small beads (fines) varied from batch to batch. The following method gave control sterile columns with uniform flow rates that did not change appreciably over a period of 12 days. Glass beads with a nominal diameter of 100 μm were boiled in a detergent (Micro, International Products Corp., Burlington, NJ, USA) known to give a very low organic residue. The beads were allowed to fall through a column of high purity water until detergent free and the fines had been removed. This took several cycles of washing and fine removal. Unless the fines were removed, control, uninoculated columns of beads steadily decreased their flow rate with time of incubation. The diameter of the beads produced in this manner was 100 $\pm$ 10 μm. A sterile suspension of beads (2 mL beads in 5 mL medium) was pipetted into a sterile 10 mL plastic syringe modified so that a 16 mm translucent cap would fit closely. The bead bed was washed with 12 mL medium and the flow rate of medium through the column measured in triplicate. This volume was needed for the column bed to pack so that flow rates did not vary as a function of the volume that had passed through the column at a later time. The initial flow rate was compared to that after incubation with microorganisms, *i.e.*, each column was its own control. Twenty four columns were prepared for each experiment. This allowed triplicate control columns (uninoculated) and seven tests. Columns of beads were inoculated with 0.5×10^6 diatoms in ASP_2 with 0.25 mM calcium and/or 0.5 mL of a bacterial suspension, absorbance 0.5–0.7. The diatoms were prepared in the medium containing lower level of calcium so that a uniform suspension was made[30] which ensured that uniform inoculation of each column. A cell count of the inoculum and a determination of its chlorophyll content allowed the chlorophyll/cell to be calculated.

2.5 Sampling of the columns

At each time point three columns were drained and soluble carbohydrate and phosphate determined. The columns were then re-filled with medium and their flow rates measured. The bead bed was then extracted *in situ* with 0.5 M $NaHCO_3$.[31] This extract was drained and its carbohydrate, together with

that left in the extruded beads, was determined. The beads in a second set of three columns were extracted with 90% acetone to determine chlorophyll *a* as an indicator of diatom growth.

2.6 Confocal microscopy

A bead suspension in HO medium contained in a 55 mm petri dish was inoculated with *Navicula* sp.1 at zero time, a second dish was inoculated at zero time with diatoms and *Pseudoalteromonas haloplanktis*. A third dish was inoculated with diatoms and after 4 d incubation this was then inoculated with bacteria. Just before microscopic examination, 3 mL of SYBR Green I was added with gentle swirling to stain the bacteria *in situ*. Confocal microscopy was then performed using a Leica microscope, model TCS-SP fitted with a 40 × water-immersible lens. Optical slice images (0.5 μm) were collected at 500–560 nm (green fluorescence) using a 488 nm excitation wavelength from an argon laser. Red fluorescence was collected at 580–680 nm using excitation from a krypton laser (568 nm). Images were analyzed by Adobe Photoshop and Imaris-3D software. Scanning electron microscopy was performed using a JEOL 6100 microscope fitted with a cryostage. Samples were coated with 10 nm of pure gold.

2.7 Staining of microorganisms

Viable and non-viable diatom cells were distinguished from one another by staining with the dye Sytox Green (Molecular Probes Inc., Eugene, OR, USA) in DMSO. Wet mounts were stained for 15 min in 10 000 × dilution of the solution provided by the manufacturer (no concentration information for the dye is given by Molecular Probes Inc.) and observed in an epifluorescence microscope using a B2A filter set (excitation filter, 450–490 nm; dichroic mirror, 510 nm; and emission filter, 520 nm; Nikon Instruments Inc.).The final concentration of DMSO in the cell suspension was 5%. Cells with compromised cell membranes stained green, viable cells were not stained. Bacteria were also stained with a 1/200 dilution in growth medium of the stock solution of SYBR Green I supplied by Molecular Probes Inc. At this dilution, no rinsing of the stained cells was needed and the background did not fluoresce. They were observed with a B2A epifluorescence filter set or in the confocal microscope.

Diatom adhesive plaques or footpads were stained either with 0.1% Acridine Orange (AO) in water for 15 min and washed with water, or Concanavalin A conjugated to fluorescein isothiocyanate and washed with growth medium. Footpads stained orange with AO and yellow–green with Con A when observed using the B2A filter cube

2.8 Production of footpads

Diatom cells were grown on the surfaces of microscope slide cover glasses, microscope slides or in small four-place bioreactor/culture slides (Becton-Dickenson, Franklin Lakes, NJ, USA) for 2 d and then treated with 10 mM EGTA, pH 7.8 or 0.2 M EDTA, pH 7.25 for 25 min.[32] The cells were washed with medium and observed with dark phase optics or after staining.

2.9 Extraction of polymers

(i) To determine the effect of the state of the diatom cells on the relative amount and quality of the polymers extracted, we extracted both fresh and lyophilized cells using 1.5 M NaCl as the extractant.[33]

(ii) Water soluble carbohydrate polymers were considered to be soluble in the growth medium and were measured in the column effluent produced by draining the column when it was sampled. Polymers soluble in 0.5 M $NaHCO_3$ at 90 °C were extracted *in situ* by adding 5 mL of the solution to the column and then draining one bed volume of liquid from the column. The fact that the pH of the effluent changed from pH 7.8 to about 8.5 showed that the column liquid was fully exchanged. The columns were incubated for 1 h at 90 °C and then drained. The remaining carbohydrate polymers were determined directly on the glass beads. These polymers can be seen microscopically (dark phase) after bicarbonate extraction as amorphous clear structures surrounding the cells. The matrix fraction could be contaminated by intracellular carbohydrate-reacting materials not removed by the previous treatment. Contamination of one fraction with another because of the volume of liquid retained by the bead bed was corrected by using a knowledge of the hold up volume.

3. Results

3.1 Growth of diatoms

We have shown previously[24] that *Amphora coffeaeformis* grows in ASP_2 medium with a generation time of 12 h at 25–28 °C. The organism can grow heterotrophically (dark growth) and mixotrophically (stimulation of light-limited growth) on D-glucose, D-fructose, L-glutamate and yeast extract. *Navicula* sp.1, on the other hand, has an autotrophic generation time of 32 h, is heterotrophic only on 0.05% yeast extract and mixotrophic on 0.5 mM D-glucose or L-glutamate.

3.2 Extraction of putative diatom polymers

The extracts of fresh and lyophilized diatoms were of very different composition. Table 1 shows that a much larger amount of both protein and carbohydrate reacting material was extracted from the lyophilized cells than from the cells in a fresh state. When samples of these cells were stained with Sytox Green and examined microscopically, the percentage with compromised membranes was found to be 11% ($n = 633$) for the fresh cells and 100% ($n = 300$) for the lyophilized cells. Microscopic examination of footpads, shown in Fig. 1, indicated that they were soluble in hot (90 °C) bicarbonate solution.

3.3 Experiments with glass bead-filled columns

(i) Diatoms alone. Fig. 2 shows that the growth of *A. coffeaeformis* on beads leads to their stabilization against a mechanical force, in this case gravity. The image was made with the bioreactor turned at 60° to the horizontal plane. In each case, the control uninoculated beads adopted the horizontal position (avalanched), whereas those with diatoms attached did not move.

Fig. 3 shows the influence of the growth of *Amphora* on the reduction in flow rate of the packed bead beds. This reduction was not evident until the phosphate in the medium had been reduced from 24 μM to about 0.5 μM. Since cells ceased increasing in chlorophyll content after 120 h, and therefore most probably in number, it is not likely that the cells themselves reduced the flow through the bioreactor. Fig. 4 indicates that reduction in flow was also not correlated with production of bicarbonate-soluble carbohydrate polymers. Neither did the production polymers soluble in the medium influence flow rate (data not shown). However, flow reduction was correlated

Table 1 Analysis of extracts from fresh and lyophilized diatom cells

	Amphora[a]		*Navicula*[a]	
Analysis	Fresh	Lyophilized	Fresh	Lyophilized
Carbohydrate	3.6	68.8	1.6	82.7
Protein	24.0	135.0	1.4	168.3

[a]Quantities are given in μg mg^{-1}dry wt. of cells.

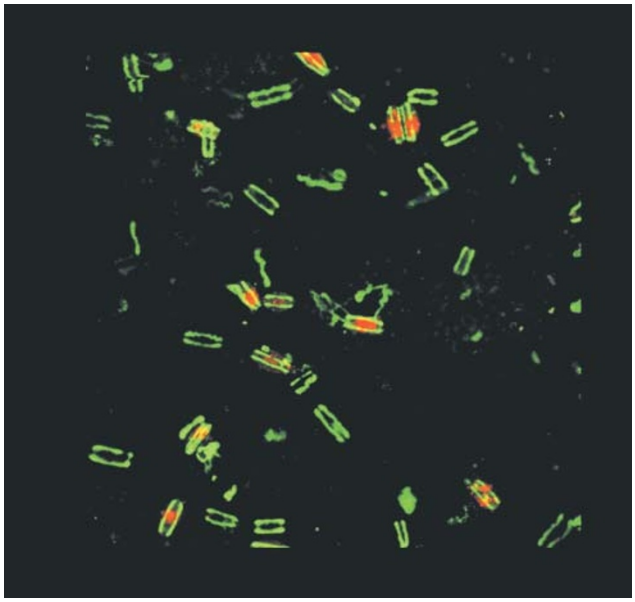

Fig. 1 Substratum-attached material ("footpads") remaining after treatment of *A. coffeaeformis* with EGTA. The footpads are soluble in hot bicarbonate solution and are stained here with Concanavalin A conjugated to fluorescein isothiocyanate (100 μg mL^{-1}, Sigma Chemical Co.). Red autofluorescence of the diatom chloroplast is seen above the footpads when diatoms were not removed from the substratum.

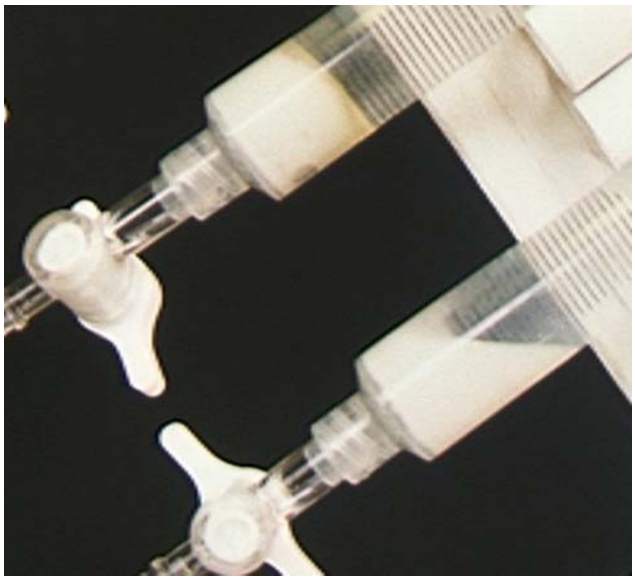

Fig. 2 Bioreactors containing glass beads (100 μm). Upper bioreactor was inoculated with *A. coffeaeformis* 10 d previously. At this time diatoms were no longer growing and matrix polymer formation was well developed. Lower bioreactor is uninoculated control. Note avalanching of beads in the uninoculated control.

($R^2 = 0.96$) with matrix polymer synthesis (Fig. 5). The results for *Navicula* sp.1 appeared similar (Fig. 6 and 7), but not identical to those for *Amphora*. Again, carbohydrate polymers soluble in the medium had no influence on the flow through the bioreactors (Fig. 7), but there was no obvious correlation with any of the carbohydrate fractions insoluble in the medium. Both matrix and bicarbonate-soluble polymers increased more rapidly after the medium became phosphate limited (120 h). In contrast to *Amphora*, the bicarbonate-soluble polymer rather than the matrix polymer fraction increased most after the phosphate had been depleted. Even when these three polymers were summed (Fig. 8), the correlation between this parameter and flow reduction was not strong.

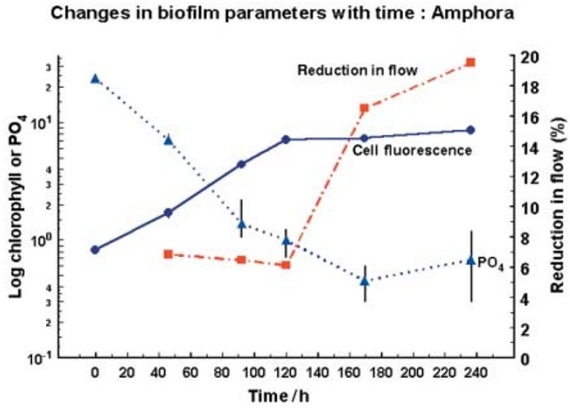

Fig. 3 Changes in biofilm parameters with time: *A. coffeaeformis*.

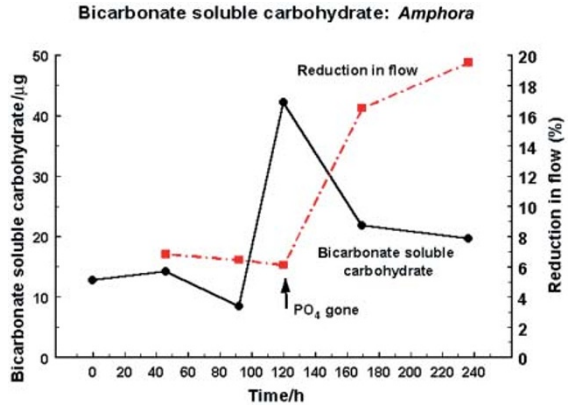

Fig. 4 Extracellular carbohydrate polymer that is soluble in bicarbonate solution: *A. coffeaeformis*.

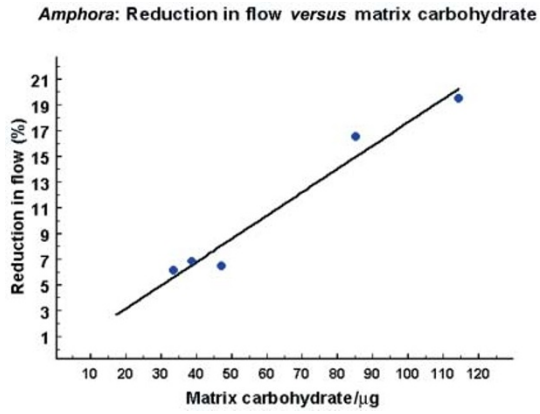

Fig. 5 Reduction in hydraulic conductivity (flow) of bioreactors during growth of *A. coffeaeformis* and concomitant production of matrix polymer. Regression coefficient, $R^2 = 0.96$. As seen in Fig. 3, this curve represents matrix polymer formation over the same period of 45–240 h.

(ii) Experiments with diatoms and bacteria in mixed cultures. The influence of bacteria from sediments on the process of bead bed stabilization was investigated using culture of diatoms alone and diatoms in admixture with the bacterium *Pseudoalteromonas haloplanktis*. We found that the presence of bacteria in a diatom culture gave different results than those obtained with diatoms alone (Fig. 9). The ability of the diatoms to reduce the hydraulic conductivity of the bioreactor bead bed was inhibited by the presence of the bacteria. However flow rates with bacteria alone were not statistically different from those in the uninoculated controls (compare columns 1 and 2, Fig. 9). The flow rates through bioreactors inoculated with

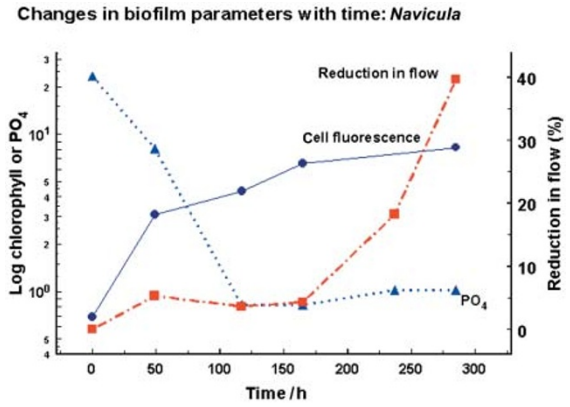

Fig. 6 Changes in biofilm parameters with time: *Navicula* sp. 1.

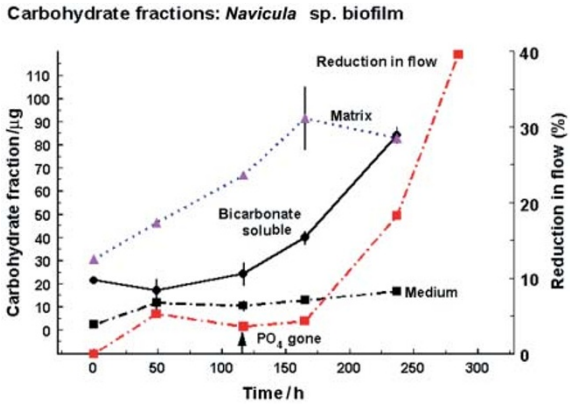

Fig. 7 Production of extracellular carbohydrate polymers with time: *Navicula* sp.1.

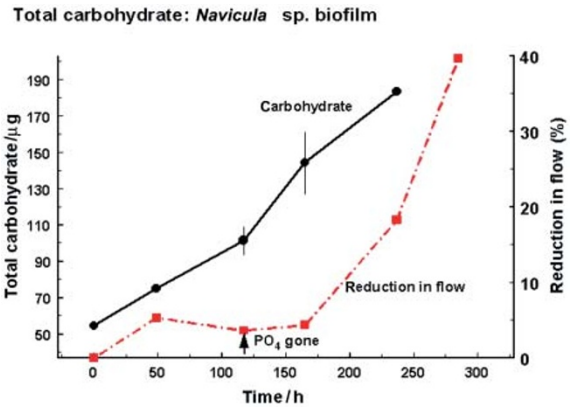

Fig. 8 Individual carbohydrate polymer fractions summed to produce a total carbohydrate value: *Navicula* sp.1.

Amphora and *Navicula* alone or a mixture of both organisms were not statistically different (compare columns 5, 6 and 7, Fig. 9). However, the flow rates with bacteria and diatoms in co-culture were greater than those obtained with a single diatom species (compare columns 3 and 6; 4 and 7, Fig. 9), but not when diatoms of both species were present (compare columns 5 and 8, Fig. 9). The relative fluorescence values for chlorophyll extracted from the bioreactors inoculated with a single diatom culture and those inoculated with diatoms plus bacteria were not different. For example, the values for *Amphora* alone and *Amphora* with *Pseudoalteromonas* were 8.65 and 8.75; the equivalent figures for Navicula were 6.45 and 6.96.

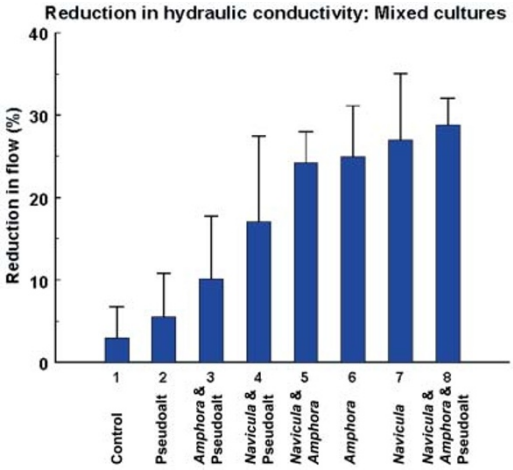

Fig. 9 Reduction in hydraulic conductivity of bioreactors inoculated with mixed cultures. Bars represent means $\pm$SD ($n = 3$).

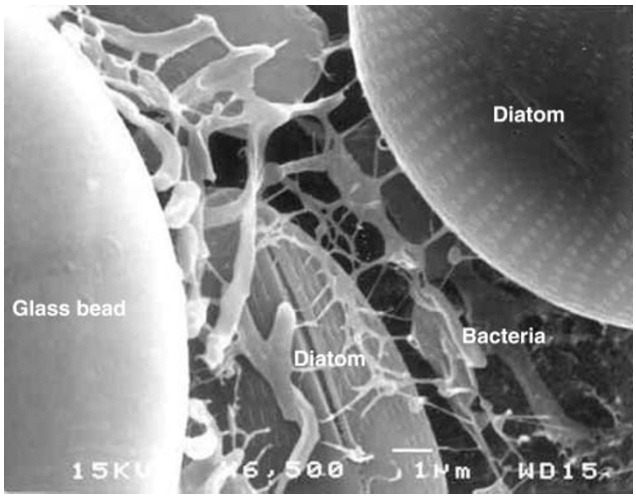

Fig. 10 Scanning electron micrograph of a mixed culture of *Navicula* sp. 1 and *P. haloplanktis* on 100 µm glass beads. (Equipment: JEOL 6100 instrument fitted with a cryostage, sample was coated with 10 nm gold).

In an effort to elucidate the reasons for these results we examined glass beads colonized with diatoms alone and those colonized with mixtures of bacteria and diatoms using both SEM and CSLM. Fig. 10 shows a typical biofilm of *Navicula* and *Pseudoalteromonas* growing on beads. The micrograph does not provide clear clues to explaining the bacterial–diatom interaction, but it does demonstrate that the bacteria have preferentially colonized the bead surface. Fig. 11 supports this idea. Panels 11(a) and 11(d) show complete coverage of the beads by *Navicula* sp.1 whereas panels 11(b) and 11(e) and 11(c) and 11(f) show that in the presence of bacteria, diatom colonization is inhibited. Bacteria in these images are green (SYBR Green 1 staining) and diatoms are red (chlorophyll autofluorescence). Note that some of the bacteria are seen as streaks showing that some of them were motile. This indicates that the stain did not kill them. Motile diatoms also showed as streaks. Such cells were seen only in the presence of bacteria [compare image 11(a) with 11(b) and (c)]. These interpretations were supported by results shown in Fig. 12(a) and (b). Images were obtained by collecting optical "slices" of the bead at its center so that only the bead circumference can be seen. The differential colonization effect between diatoms and diatoms with bacteria is obvious. In admixture, bacteria colonize the beads preferentially.

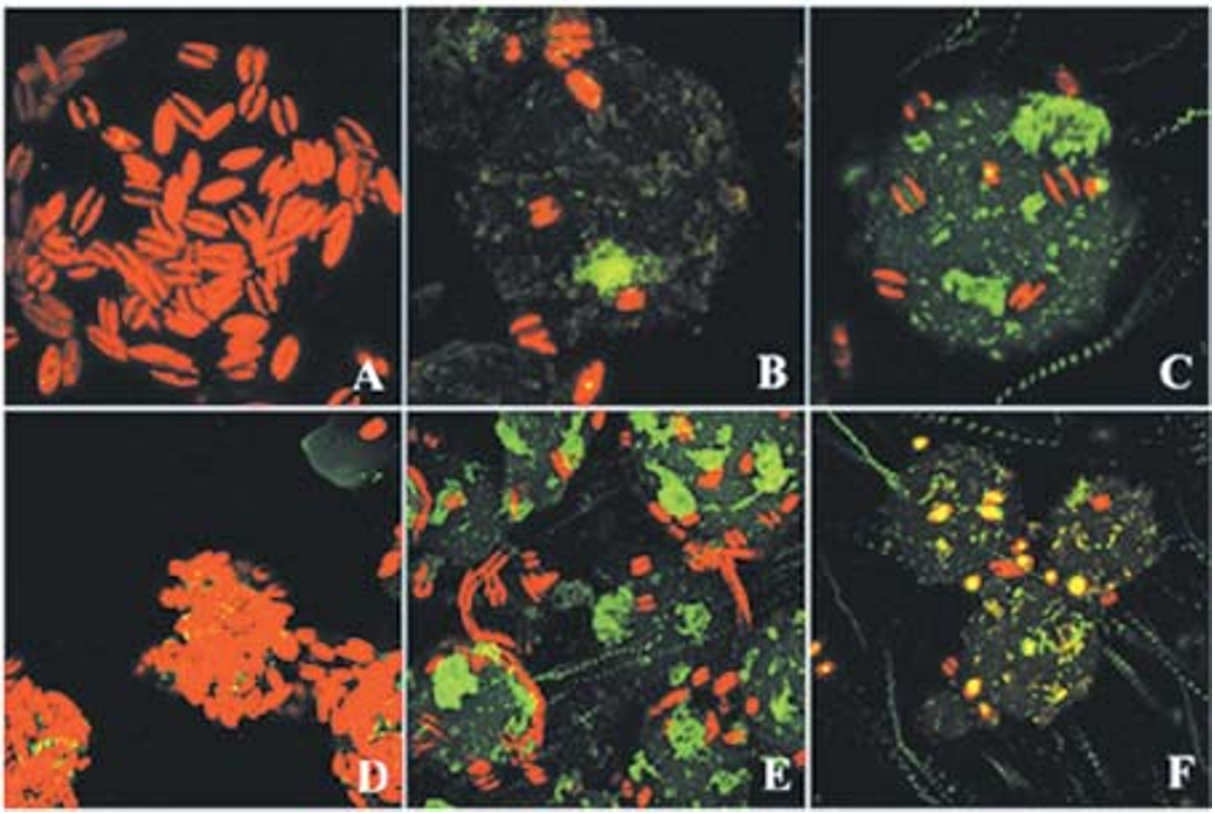

Fig. 11 Confocal microscope images of colonized glass beads. Diatoms (*Navicula* sp. 1) are seen by the red fluorescence of their chloroplast. Bacteria (*P. haloplanktis*) are stained with SYBR Green 1. (a) and (d) Diatoms alone; (b) and (e) *P. haloplanktis* added to the diatom culture after 4 d; (c) and (f) *P. haloplanktis* added to diatom culture at time zero. Upper panel magnification was 1000×, lower panel magnification was 2300×.

4. Discussion

There is general agreement that the metabolic activities of the benthic diatom community influence the physical stability of marine sediments and that extracellular carbohydrate polymers synthesized by diatoms play an important role in the process.[3,5,22] However there is disagreement concerning the details of the stabilization process and which extracellular polymers are involved, *e.g.*, ref. 22. We suggest that much of the disagreement can be traced to differing methodologies between laboratories and what appears to be a tacit assumption that all diatom species behave similarly. If they do not, field investigations where speciations of the biofilm populations differ will not agree. For instance, extracellular carbohydrate polymers may well be extracted from one species of diatom with a certain efficiency and a differing efficiency from another species. Further complication is that diatoms of a single species secrete polymers differing in structure, depending on the growth phase.[34,40] A sediment will contain many species in various stages of the cell cycle. The fact that most diatoms secrete increased levels of extracellular polymer in the transition to stationary phase is however agreed, as is the influence of phosphate limitation on this process. Phosphate limitation stimulates ''overflow metabolism'' which results in increased polymer secretion (ref. 17 and references therein). Our argument is applicable to any illuminated biofilm, not just those on sediments.

Our results with lyophilized and fresh cells (Table 1) support the idea that the lyophilization process causes cell lysis and thus makes the intracellular polymers available to solvents. Cells examined microscopically before and after lyophilization showed very little difference with light or dark phase optics, but were clearly different when viewed with epifluorescence optics after being stained with a cell membrane impermeant dye. Both Underwood *et al.*[33] and Staats *et al.*[36] discuss the possibility that intracellular polymers, notably chrysolaminarin—a β 1-3 glucan, were extracted from lyophilized cells using solutions of EDTA, NaCl or water alone. Both groups conclude from indirect evidence that intracellular polymers are not extracted. Neither group however monitored membrane integrity. Underwood *et al.*[33] did show that the yield of colloidal polymer from sediments was increased two-fold after lyophilization. Most investigators routinely lyophilize sediment samples to preserve the integrity. In the light of our results we suggest that perhaps the extraction process should be re-evaluated. de Brouwer *et al.*[16] have made an unpublished observation that lyophilization causes cell lysis in diatoms.

We have measured three operationally defined extracellular carbohydrate polymers. These are: (i) the polymer easily soluble in artificial seawater (24 ppt), (ii) the polymer secreted by stationary cells before they adhere permanently and which is not soluble in saline medium but is soluble in hot bicarbonate solution; and (iii) the polymer remaining after bicarbonate soluble polymers have been removed. Fraction (i) will contain polymer secreted as a result of motility; (ii) represents the water insoluble, bicarbonate soluble polymers referred to as WIBS by Wustman *et al.*[31]; and (iii) represents the matrix polymer visibly responsible for biofilm architecture and most likely the product of ''overflow metabolism''.[17] There is a potential for this fraction to contain also any intracellular carbohydrate-reacting material not extracted earlier. The WIBS fraction has been investigated by Wustman *et al.*[31] in detail. The sugar constituents of this fraction from *Amphora coffeaeformis* and *Achnanthes longipes* did not differ appreciably in either sugar content or their amounts, as determined by GC-MS analysis. However, mechanically isolated polymers from *Amphora* differed considerably in composition from the WIBS fraction of the same organism and from the mechanically isolated stalks of *Achnanthes*. Although we have no comparable information on *Navicula*, it seems reasonable to suggest that extracellular

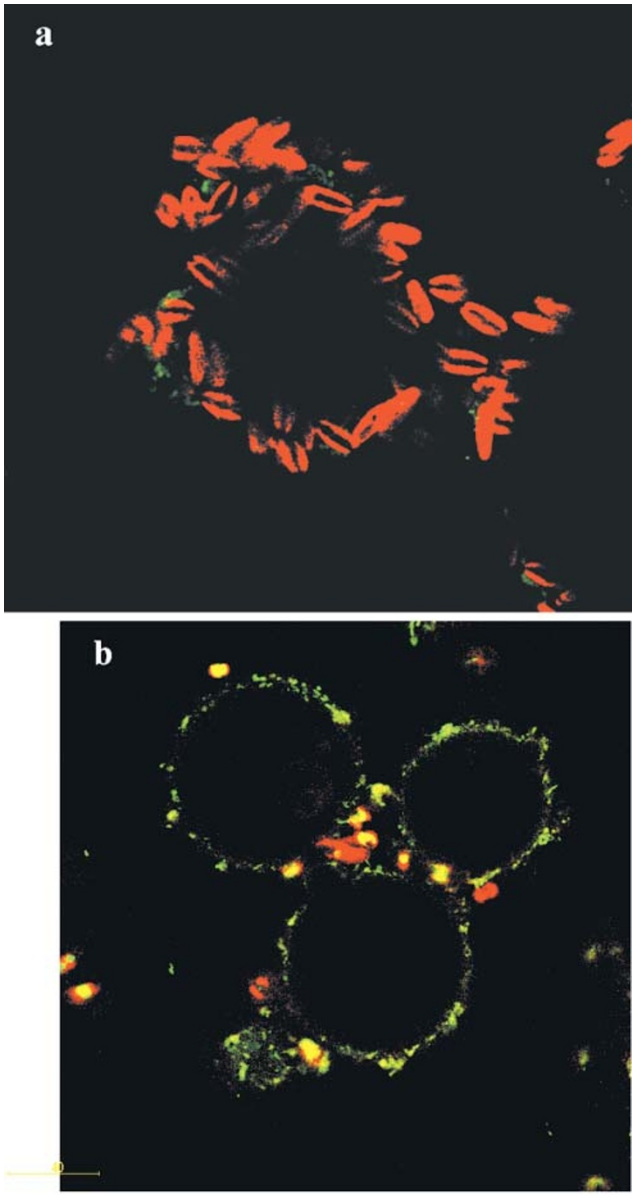

Fig. 12 Confocal microscope images of colonized glass beads. (a) *Navicula* sp.1; (b) *Navicula* sp.1 and *P. haloplanktis*. Both (a) and (b) are optical "slices" through the centre of the beads, thus cells are seen on the periphery of the beads.

polymers of this organism could differ from those of *Amphora*. This is suggested by the time courses of accumulation of polymers in this fraction (*cf*. Fig. 4 and 7).

The mere presence of diatom cells on the surfaces of the glass beads in the bioreactors did not interfere with flow through the bead bed or influence its physical stability. With both *Amphora* and *Navicula*, the reduction in flow and stabilization of the bead bed (Fig. 2) did not occur until phosphate concentration in the medium fell below an assimilable level and growth, as measured by chlorophyll fluorescence, ceased. The fact that growth measured by this parameter ceased when phosphate became limiting allows confidence in the use of chlorophyll as a biomass level indicator. It can be seen from Fig. 2, 3 and 5 that the stabilization of the bead bed, the reduction of its hydraulic conductivity and the production of matrix polymer occurred in the same time-frame.

Our results with glass bead-filled bioreactors do not support the involvement of growth medium-soluble polymers in sediment stability because this polymer fraction did not change throughout the course of the diatom growth. Its amount was not correlated with the reduction in hydraulic conductivity of the bead bed. In this respect, our results agree with those of Staats *et al.*[36] and van Duyl *et al.*,[37] but not those of Underwood *et al.*[33] or Patterson.[35] Staats *et al.*,[36] used both axenic cultures of *Cylindrotheca closterium* and mudflat samples to measure exopolysaccharide secretion in various conditions. Their results did not support the involvement of the motility polymer in the accumulation of exopolysaccharide in the light. The results of Underwood *et al.*[33] support their involvement. To our knowledge, no one working in the field of microbial sediment stabilization has suggested that a bicarbonate-soluble diatom exopolymer component contributes to the stabilization process. Our choice of this fraction was because Wustman *et al.*[31] stated that "…the majority of the adhesive polymers were contained in this fraction…" In *A. coffeaeformis* we found that this fraction did not contribute to sediment stabilization as measured by our flow method. It is important to realize that in the early stages of growth, this fraction probably contained internal carbohydrates in addition to those from the extracellular environment, but that this is less likely in later growth stages once the cells had become phosphate limited. Staats *et al.*[17] showed that intracellular carbohydrate polymers did not increase during phosphate limitation. The fraction responsible for stabilization appears to be the matrix polymer. This polymer would appear in the total carbohydrate of Underwood *et al.*[33] Our results suggest that its accumulation is controlled by the level of phosphate in the medium. This type of response in diatoms is well known (refs. 17, 38, 39 and references therein).

The results of identical experiments with *Navicula* sp.1 indicate that it is unwise to generalize based on results with a single organism. Reduction in flow occurred when phosphate concentration limited growth, but the WIBS fraction and the matrix polymer were produced throughout the growth period, as well as in the stationary phase, although the WIBS production did increase at phosphate limitation (Fig. 7). It can be seen from Fig. 8 that indeed the production of total extracellular carbohydrate polymers was correlated with reduction in flow through the bioreactors but that the correlation was not as clear cut as with *Amphora*. Smith and Underwood[40] using pyrolysis mass spectrometry, also showed that diatom EPS differs from species to species and, depending on the phase of growth, within species.

In view of the known interactions between diatoms and bacteria,[23] our initial hypothesis concerning mixed species biofilms and sediment stabilization was that it was likely that there would be a positive or may be a synergistic interaction leading to an enhanced sediment stabilization. Our results do not support this hypothesis (Fig. 9). The presence of *P. haloplanktis* and diatoms actually increased the flow through the bioreactors over that found with diatoms alone. This was true for either *Amphora* or *Navicula* with bacteria, but not true when *both* diatom species and bacteria were combined. It was considered possible that the results with single diatom species and bacteria could arise from an inhibition of diatom growth and/or metabolism by the bacteria. However, as seen by chlorophyll *a* analysis, we found that there was no inhibition of diatom growth. Note that under the conditions of these experiments, the diatoms and the bacteria could utilize the organic substrates in the medium. It is not clear why, when two diatom species and the bacteria are grown together, the flow rate is not different from that obtained in the absence of the bacteria. Van Duyl *et al.*[15,37] showed that in natural sediments, the activities of heterotrophic bacteria were coupled to the production by diatoms of extracellular carbohydrate polymers. This activity, which they suggest was mediated by β-glucosidase, was dependent on the presence of water-soluble, but not water-insoluble, carbohydrate polymers. These authors also suggest that the utilization of the extracellular polymers by bacteria and the concomitant production of bacterial EPS could also contribute to sediment stabilization. Our results do

not support this, but another speculation by the same authors, *i.e.*, rapid reduction of extracellular carbohydrate pools by bacteria, could be a destabilizing force in already stable sediments. For instance, Fig. 10 shows very little extracellular polymer in the interstitial spaces of the beads. In addition, the CLS micrographs (Fig. 11 and 12) show distinctly the differential colonization pattern for diatoms alone and diatoms in the presence of bacteria. Preliminary experiments have shown that marine sediment bacteria produce in the growth media materials that inhibit colonization of surfaces by diatoms. Gawne *et al.*[41] have shown that the story concerning bacterial influences on colonization patterns of *Achnanthes longipes* is highly complex. Elucidation of the means by which *P. halopklanktis* can control diatom colonization patterns is the focus of our continuing study. The implications for this work are not limited to the marine environment. Battin and Sengschmitt[42] indicate that similar microbial phenomena can operate in rivers.

5. Conclusions

For the diatom and bacterial species we have studied, we conclude that: (i) Diatom matrix extracellular carbohydrate polymer is largely responsible for sediment stabilization and thus biofilm architecture; (ii) soluble diatom motility polymer plays no part in the sediment stabilization process; (iii) possibly because of their lytic activities on exopolymers elaborated by diatoms, bacteria are more likely to inhibit than enhance diatom driven sediment stability; and (iv) it is unreasonable to make general predictions based on the results obtained with one, or perhaps several, organisms.

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