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Bacterial-algal interactions in polysaccharide production

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ABSTRACT: The effects of phosphate limitation and of the presence of marine bacteria during phosphate limitation on growth and polysaccharide production in the diatom *Cylindrotheca fusiformis* grown in batch cultures are described in this paper. Growth of *C. fusiformis* was inhibited under low Pi (inorganic phosphate) conditions, corresponding to an increasing N/P ratio, and higher amounts of polysaccharides were extruded in the medium, in particular during the stationary phase of growth. The presence of bacteria reduced phytoplankton cell density only when the phosphate added corresponded to 1/6 of the initial amount. Even when diatom cell growth was not affected, the presence of bacteria stimulated a higher polysaccharide production. These results are interpreted in the light of the fact that nutrient-stressed phytoplankton cells produced and released a higher amount of polysaccharides and, as bacteria exhibited a better utilization of phosphate than algal cells, their presence accentuated the Pi depletion, resulting in a higher polysaccharide production.

KEY WORDS: Bacteria · Diatom · Growth · Marine snow Polysaccharides

INTRODUCTION

Phytoplankton cells are known to release polysaccharides in the external medium; this exudation is highest during the stationary phase of growth and increases under nutrient limitation (Myklestad 1977). During natural phytoplankton blooms, extracellular algal products can stimulate bacterial growth and activity, leading to the complete mineralization of algal material (Bell et al. 1974). Azam & Cho (1987) have hypothesized that phytoplankton exudation has evolved as a mechanism to establish mutualism with bacteria: the polysaccharide layer becomes enriched with dissolved organic nitrogen and phosphorus while bacteria have hydrolytic enzymes which can hydrolyze the polymers on the algal cell surface. However, depending on the nutritional status of the environment, bacteria can play variable roles in that they can also compete for nutrients with phytoplankton (Azam et al. 1983): at high nutrient levels, bacteria become remineralizers, but they compete with phytoplankton at low nutrient levels (Tupas & Koike 1990). Nutrient-stressed algae secrete more polysaccharides; in this way they try to maintain the mutualism, but they aggregate and sink; bacteria colonize the mucus, rendering it even more sticky, their metabolism becomes heterotrophic and most cells die (Azam & Smith 1991).

Obernosterer & Herndl (1995) found that low Pi (inorganic phosphate) conditions rather than N depletion leads to an increase in the photosynthetic extracellular release which is not efficiently utilized by bacteria. These in fact exhibit higher α- and β-glucosidase activities under P limitation than under N limitation. In recent years the Adriatic Sea experienced the appearance of mucilage which was considered to be of diatom origin covering a very large area; in 1991 the occurrence of marine snow coincided with a high N/P ratio (Kaltenböck & Herndl 1992).

To further test the hypothesis of Azam & Smith (1991) we cultured cells of the diatom *Cylindrotheca fusiformis*, a species very common in the Adriatic Sea, both in axenic conditions and in the presence of marine bacteria, and both conditions were tested with varying N/P ratios. Previous studies on the relationships between algae and bacteria were usually per-

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formed by measuring bacterial growth and production either *in situ* or in microcosms. The present work is mainly focused on the metabolic responses of phytoplankton cells, measured as growth rate and polysaccharide production, to the presence of bacteria and to nutrient limitation. In particular, we wanted to distinguish carbohydrates that are exuded in the environment from those which constitute a cellular reserve.

MATERIALS AND METHODS

Organisms and culture conditions. *Cylindrotheca fusiformis* Reimann and Lewin was isolated from natural phytoplankton communities in coastal waters of Emilia-Romagna, Italy. Cultures were maintained axenically in f/10 medium at 20°C under a 16:8 h light:dark cycle (ca 1600 µW cm^{-2} from cool, white lamps) (McLachlan 1973). For experimental work the cultures were transferred to f/2 medium (Guillard & Ryther 1962) and axenically grown under the same conditions in sterile 250 ml Erlenmeyer flasks sealed with cotton plugs. Cell counts were made every other day in settling chambers by the Utermöhl (1931) method. Before each experiment the cultures were treated with the following mixture of antibiotics: 250 µg penicillin G, 250 µg kanamycin, 500 µg streptomycin sulfate per ml culture, then checked for sterility on ZoBell 2216 agar plates.

Eight different bacterial strains were isolated from sediments collected by means of a bucket in the Northern Adriatic Sea and maintained in ZoBell medium 2216 (ZoBell 1941). Preliminary experiments were performed with all the different bacteria but, as results were very similar, most of the work was continued with a strain called SM7, composed of Gram-positive, coccoid cells. This strain was chosen because the cells were large enough to be observed under light microscope at 100×.

Three different nutrient conditions were adopted, corresponding to 3 different N to P atomic ratios: complete f/2 medium (control cultures; N = 883 µM, P = 36.3 µM, N to P ratio = 24), f/2 with a Pi content equal to 1/3 of control medium (indicated as 1/3 P, N to P ratio = 73), and f/2 with a Pi content equal to 1/6 of control medium (indicated as 1/6 P, N to P ratio = 146). These nutrient conditions were used to grow monospecific cultures of *Cylindrotheca fusiformis* and SM7, or mixed cultures of bacterium and algae.

The mixed cultures were obtained by adding 1 ml bacterial cells, exponentially growing in ZoBell medium, to cultures of *Cylindrotheca fusiformis* immediately after subculturing them in fresh medium. This consisted of 150 ml f/2 medium to which diatoms were added at an initial density of 2000 to 3000 cells ml^{-1}.

Bacterial growth in monospecific cultures was followed spectrophotometrically at 750 nm (Jasco 7800), while growth of bacteria in mixed cultures was followed by cell counting with a Neubauer chamber.

Carbohydrate analysis. Carbohydrates were analysed by the phenol-sulphuric method of Dubois (Dubois et al. 1956) using glucose as a standard. In particular, total carbohydrates were measured by adding 50 µl 80% phenol and 5 ml 95% sulphuric acid to 1 ml culture supplemented with 1 ml filtered seawater (salinity 25‰). The extracellular carbohydrate fraction was analyzed as previously described (Pistocchi et al. 1997); briefly, 5 ml cultures were acidified with 20 µl 1 N HCl and centrifuged at 10200 × *g* for 10 min in a Beckman J2-HS centrifuge (rotor JS-13.1) to pellet the cells, then 2 ml of the supernatant was analyzed by the phenol-sulfuric acid method.

For each culture condition there were 2 replicate flasks and every experiment was repeated 2 or 3 times. Values presented in the figures are the means ± standard deviations (SD) of the different experiments, each consisting of duplicate or triplicate samples.

Pi uptake. Monospecific or mixed cultures were established in flasks containing 150 ml of the 3 different media, as already described, and grown up to Day 7. Every day at the same time (10:00 h) 1 ml culture for cell counting and 10 ml for filtration through GF/C Whatman filters were withdrawn. Pi content on the filtrate was determined according to Strickland & Parsons (1972).

RESULTS

Cell growth

Growth of *Cylindrotheca fusiformis* in 3 different nutrient conditions (described in 'Materials and methods') was followed in the presence or in the absence of bacteria (Fig. 1a–c). Growth of axenic *C. fusiformis* cultures was affected by Pi limitation in that, in the media containing 1/3 and 1/6 P, the final cell number per ml culture was reduced by 39 and 73%, respectively, compared to nutrient replete cells. The presence of bacteria, under the same nutrient conditions, did not alter the final cell concentration of control cells and of cells grown in 1/3 P, while the condition with the highest Pi limitation (1/6 P) resulted in a 75 to 80% lower cell density compared to cultures grown with the same Pi concentration but without bacteria (Fig. 1c).

From the growth curves it appeared that, in the presence of bacteria, diatom growth during the exponential phase was enhanced. As can be better observed in Fig. 2 the increase in diatom cell number occurred, under all culture conditions, before the onset of the

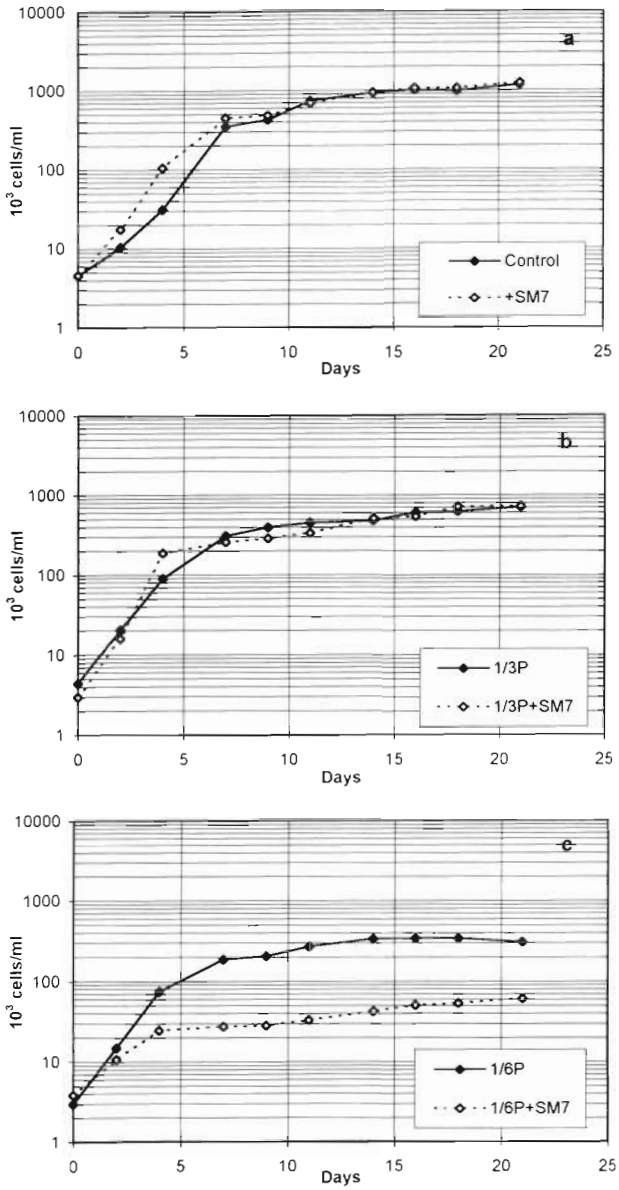

Fig. 1. Growth curves of *Cylindrotheca fusiformis* in the absence (◆) or presence (◇) of bacterium SM7 (a) in nutrient replete medium, (b) in medium with a Pi content reduced to 1/3 and (c) in medium with a Pi content reduced to 1/6 of control

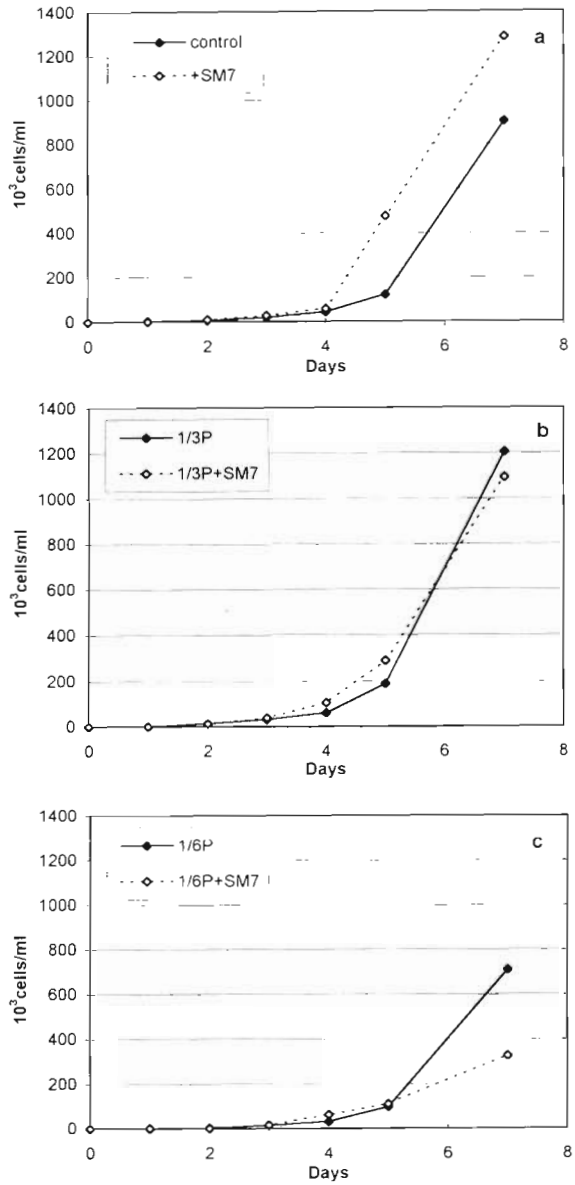

Fig. 2. Growth of *Cylindrotheca fusiformis* up to Day 7, tested as described in Fig. 1 but measured daily

fastest growth rate, during which nutrient limitation had the strongest effects. This was confirmed also by calculating the growth rate (divisions d^{-1}) (Table 1), which was always higher in the presence of bacteria except for the highest Pi limitation where the inhibitory effect was very marked.

Growth of bacteria in ZoBell medium was higher than in f/2; in the latter medium an increase in cell number, with a maximum at Day 4, for all nutrient conditions was observed. The presence of algae caused a higher concentration of bacterial cells per ml, especially in the media containing 1/3 P and 1/6 P. Under these conditions bacterial cell number was nearly double that in control cultures (data not shown).

Polysaccharide production

Carbohydrate production per cell, in axenic phytoplankton cultures (Fig. 3), paralleled growth and was highest in the late stationary phase (Day 21). The total amount of carbohydrates (Fig. 3a) produced increased

Table 1. Growth rate factor (divisions d^{-1}) of *Cylindrotheca fusiformis* in the absence or presence of bacterium SM7 in 3 different nutrient conditions (described in 'Materials and methods')

Culture conditions	*C. fusiformis*	*C. fusiformis* + SM7
Control medium	0.89	1.13
1/3 P	1.09	1.49
1/6 P	1.16	0.72

with increasing Pi depletion and the same was observed for polysaccharides measured in the extracellular fraction (Fig. 3b); this fraction can be regarded as a polysaccharide fraction in that up to 70–80% of it consists of polymeric residues (Pistocchi et al. 1997). The addition of bacteria to phytoplankton growing in complete f/2 medium, while not affecting final cell density, caused an increase in carbohydrate content: the increase was highest at Day 14 (Figs. 4a & 5a, grey and black bars) for the total fraction and at Day 21 for the extracellular one (Figs. 4b & 5b, grey and black bars). The same figures also show the combined effect of the presence of bacteria and Pi depletion on carbohydrate production: in mixed cultures the amount of total carbohydrate produced was higher than that measured in axenic cultures and the increase in Pi depletion, from 1/3 (Fig. 4a) to 1/6 (Fig. 5a), enhanced this effect, with the exception of Day 21. The increase in the extracellular polysaccharide fraction was even higher and appeared in all growth phases (Figs. 4b & 5b).

Total and extracellular polysaccharide production by bacteria, grown alone in all the different media, was also measured but it always fell below the detection limit.

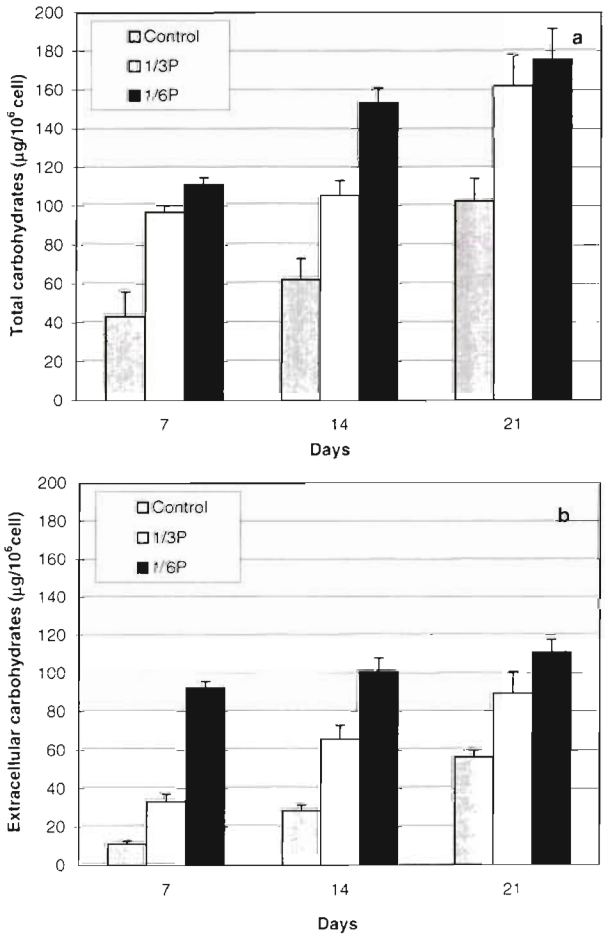

Fig. 3. (a) Total and (b) extracellular carbohydrates produced by axenic *Cylindrotheca fusiformis* cultures in nutrient replete medium and in medium with a Pi content reduced to 1/3 and 1/6 of control

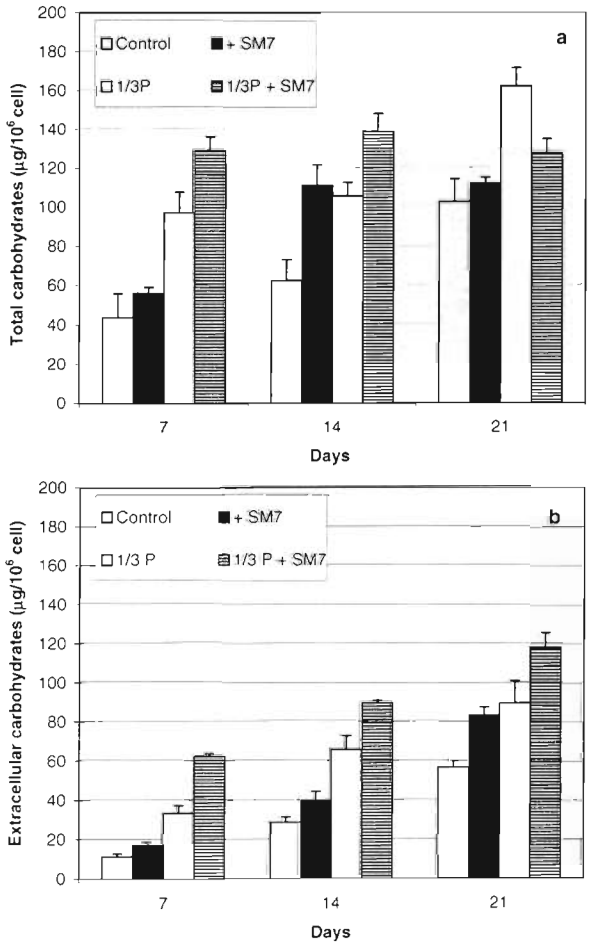

Fig. 4. (a) Total and (b) extracellular carbohydrates produced by *Cylindrotheca fusiformis* in nutrient replete conditions in the absence (grey bars) or in the presence (black bars) of bacterium SM7, and in medium with a Pi content reduced to 1/3 of control in the absence (white bars) or presence (hatched bars) of SM7

Phosphate uptake

Pi uptake from different f/2 dilutions in which phytoplankton or bacteria were grown alone or in association was also measured in an attempt to check if the 2 species competed for the nutrient. Pi uptake rates by the phytoplankton population increased with increasing Pi limitation so that Pi content was exhausted in control medium by Day 7 (Fig. 6a) and in Pi-stressed cultures by Day 5 (Fig. 6b, c). The presence of bacteria resulted, as expected, in competition for the nutrient between the 2 organisms so that phosphate exhaustion occurred earlier. Competition was particularly evident at 1/6 P in that Pi content decreased by about 90% by Day 2 (Fig. 6c). Bacteria grown alone in f/2 medium utilized phosphate up to a certain extent after which they seemed to remineralize it (Fig. 6a–c). Pi uptake by bacteria was also enhanced by Pi limitation and was faster than the uptake by phytoplankton; mixed cultures displayed an uptake rate similar to that of bacterial monocultures.

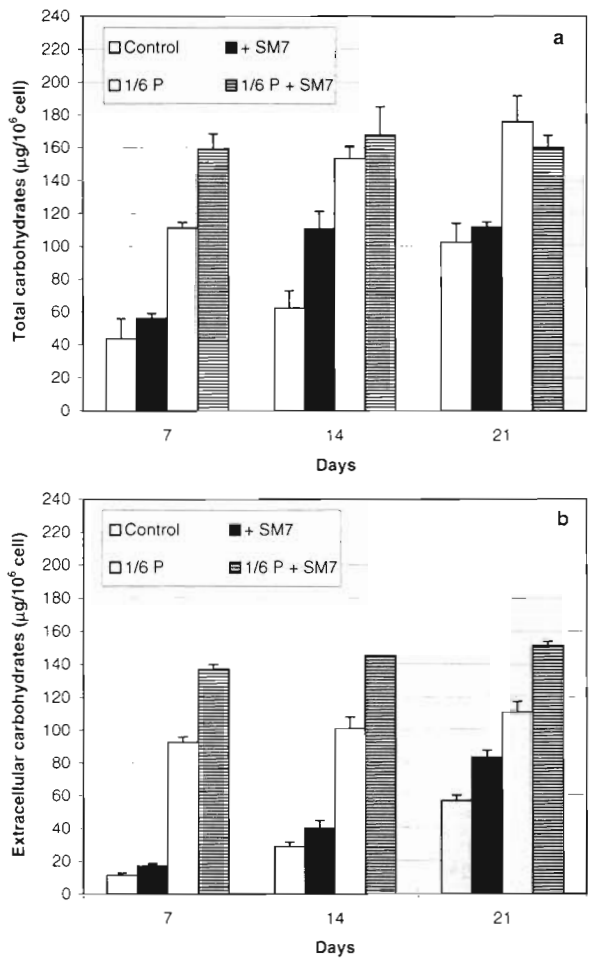

Fig. 5. As in Fig. 4 except that the comparison is between nutrient replete medium and medium with Pi content reduced to 1/6 of control

DISCUSSION

The effects of Pi limitation in *Cylindrotheca fusiformis* resulted in a reduction of both growth rate and cell density, as already observed for other diatoms (Myklestad 1977). Growth of *C. fusiformis* under Pi limitation was also studied in the presence of marine bacte-

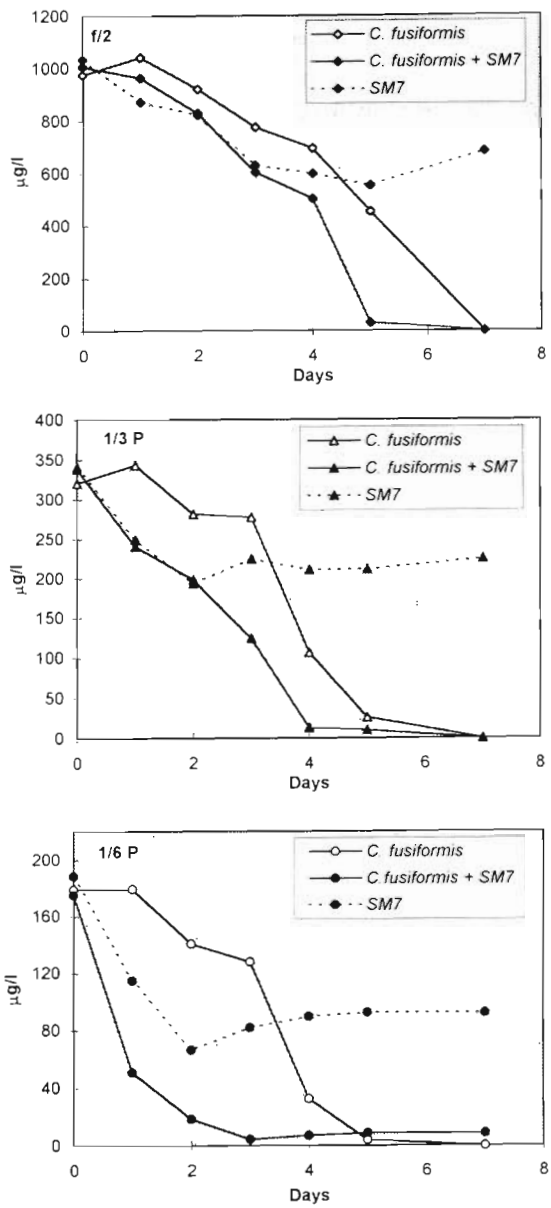

Fig. 6. Amount of inorganic phosphate in *Cylindrotheca fusiformis* axenic cultures, SM7 monocultures and in bacterium-algae mixed cultures, in the presence of 3 different nutrient conditions (f/2; 1/3 P; 1/6 P) up to Day 7 of growth

ria and it was observed that initial growth rate was slightly enhanced by the presence of bacteria under all nutrient conditions. It is difficult to explain this effect in that we cannot exclude the possibility of nutrient carry-over from ZoBell medium when diatoms were inoculated with bacteria. An alternative explanation could be that during the first period of growth, when competition for the nutrient between the 2 microorganisms is not yet dramatic, phytoplankton cells benefit from the remineralization of dissolved organic matter accomplished by bacteria cells. With the progression of growth, the curves obtained in the presence or in the absence of bacteria were very similar, with the exception of the cultures grown in the presence of 1/6 P (N/P = 146); in this condition *C. fusiformis* grew less and final cell number was only 20% that of cells grown under the same Pi limitation but in the absence of bacteria.

Phytoplankton cells also stimulated bacterial growth; this effect was also observed by Bell et al. (1974) and it was explained by the utilization of algal extracellular products. In our case polysaccharide production by phytoplankton cells in the earliest phase of growth was very low, in fact it was undetectable by our assay method. However, as the detection limit was around 12 µg ml^{-1}, we cannot exclude that even the presence of a low amount of polysaccharidic substances could enhance bacterial growth.

By increasing Pi deficiency, *Cylindrotheca fusiformis* produced a higher amount of polysaccharidic substances especially during the stationary phase of growth, as previously described for *Chaetoceros affinis* (Myklestad & Haug 1972, Myklestad et al. 1989). It is worth noting that the release of polysaccharides in the extracellular medium also increased in this condition; this behaviour is relevant in that unbalanced nutrient conditions are frequently found in the Adriatic Sea and an important ecological consequence of this fact could be the production of a higher amount of mucilage per cell. Kaltenböck & Herndl (1992) reported that in 1991 the occurrence of marine snow in the Adriatic Sea coincided with a N to P atomic ratio of 70, which is very close to the ratio used in our 1/3 P treatment. However, when Pi limitation increases (as it did in our 1/6 P nutrient treatment) the occurrence of mucilage could be limited by the reduced algal growth.

In the present paper, the effect of a high N/P ratio combined with the presence of bacteria on polysaccharide production is also reported. When bacteria were also present, the phytoplankton cultures displayed a higher carbohydrate amount than when cells were grown in the same nutrient conditions but axenically. Unfortunately it was technically impossible to exclude polysaccharidic substances of bacterial origin in our determinations. However, when we measured the polysaccharide production by bacteria alone the amount was undetectable under all the conditions tested. This suggested to us that usually bacteria contribute very little to the accumulation of carbohydrates, especially if we consider the carbohydrates exuded extracellularly. The increase in the extracellular polysaccharide fraction of mixed cultures was very marked if compared to the amount produced by the nutrient replete cultures. The highest polysaccharide content was found in cultures grown with the greatest Pi limitation, where the amount of carbohydrate produced per cell was 8-fold that of controls (nutrient replete cells plus bacteria) as early as Day 7.

Data on phosphorus content showed that in monospecific cultures utilization of this nutrient was higher for bacteria than for algal cells; in mixed cultures with the greatest Pi limitation, phosphate exhaustion occurred very rapidly in that by Day 2 all the nutrient added was depleted. The superior efficiency of bacteria in assimilating phosphate has been demonstrated in previous studies with associations of algae and bacteria (Currie & Kalff 1984, Berman 1985), while Jansson (1993) demonstrated that, at extremely low concentrations of phosphate, the bacterium *Pseudomonas* was much more efficient than the green alga *Scenedesmus quadricauda* especially when the energy source was supplied solely by algal excretion.

The conclusions we can draw from these results are that during Pi limitation the growth of *Cylindrotheca fusiformis* is inhibited and its metabolism is shifted towards polysaccharide production. Part of this production is released externally and may serve to create an environment richer in organic substances which could trap the few nutrients present in sea water or withhold microbes which accelerate nutrient remineralization. This is supported by the observation made by Rinaldi et al. (1995) that in mucilage samples nutrient concentrations were usually higher than in the surrounding environment and by the observation that bacteria are abundant in marine snow (Simon et al. 1990). As bacterial cells have a higher efficiency in phosphorus assimilation than algal cells, their metabolism accentuates the Pi deficiency, and thus their presence stimulates algal cells to produce higher amount of polysaccharides. Cultured diatoms, such as the mucilage producer *C. fusiformis*, were shown to respond metabolically to the presence of bacteria as hypothesized by Azam & Smith (1991) on the basis of bacterial metabolism in mucilage samples. This fact could imply that the presence of bacteria, while not affecting phytoplankton growth, can stimulate a higher production and extrusion of polysaccharidic substances which are resistant to degradation (Smith et al. 1992) and therefore accumulate in the marine environment.

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