Bacteria–algae association in batch cultures of phytoplankton from a tropical reservoir: the significance of algal carbohydrates

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SUMMARY

1. The dissolved organic matter, especially carbohydrates, released by phytoplanktonic organisms may be ecologically important, through its influence on carbon cycling and microbial diversity. Here axenic cultures of three phytoplanktonic species, *Cryptomonas tetrapyrenoidosa* (Cryptophyceae), *Staurastrum orbiculare* (Zygnematophyceae) and *Thalassiosira duostra* (Bacillariophyceae), were inoculated with a microbial community from the same habitat in which the algae had been isolated (a tropical reservoir). Replicate cultures were not inoculated.
2. In both axenic and co-inoculated cultures, phytoplanktonic density and extracellular carbohydrate production were monitored microscopically and by high performance liquid chromatography with a pulse amperometric detector, respectively. Bacterial population density was also monitored by epifluorescence microscope in the microbial co-inoculated cultures.
3. Both bacterial and phytoplanktonic densities increased for 11 days in all cases. The use of extracellular carbohydrates by bacteria was also showed for all phytoplanktonic species. Of the three species of phytoplankton, only *T. duostra* had a faster population growth in the presence of bacteria, and reached a higher biomass than in axenic culture.

Keywords: association, bacteria, carbohydrate, culture, phytoplankton

Introduction

Phytoplanktonic organisms release significant amounts of dissolved organic carbon (DOC) to the water column (Jensen, 1983; Myklestad, 1995; Giroldo & Vieira, 2005). A wide variety of compounds are found in the DOC pool, mostly consisting of organic acids, aminoacids, peptides and many carbohydrates in both free and polymeric forms (Hellebust, 1974). The use of some of these compounds by bacteria is well known, particularly organic acids (Merret & Lord, 1973; Sundh, 1992), aminoacids (Fallowfield & Daft, 1988; Rosenstock & Simon, 2003) and monosaccharides (Jørgensen, 1990; Jørgensen & Jensen, 1994). The use of phytoplanktonic extracellular polysaccharides (EPS) as substrates by bacteria has not yet been sufficiently studied, however (Freire-Nordi & Vieira, 1996; Giroldo, Vieira & Paulsen, 2003; Colombo, Vieira & Moraes, 2004).

The phytoplankton releases organic compounds that enhance the activity of heterotrophic microbes and form a microhabitat, known as phycosphere, where bacterial-algal associations are more abundant than in the rest of the water column (Bell & Mitchell, 1972). Many authors have studied the specificity of such bacterial-algal associations (Bell & Mitchell, 1972; Bell & Sakshaug, 1980; Delong, Franks & Alldredge, 1993; Grossart *et al*., 2005). The possibility of mutualisms involving reciprocal benefits to phytoplankton

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and DOC-associated bacteria, such as the heterocyst/bacteria associations described by Paerl (1978), has long intrigued phycologists. Despite interesting hypotheses based on nutritional return mechanisms (Wood & Van Valen, 1990; Passow, Alldredge & Logan, 1994), very little is known about the possible benefits to algae in such associations, or whether the different organic compounds released by phytoplankton select for specific bacteria. On the other hand, the inhibition of algal growth by bacteria has been described extensively in the literature (Manage, Kawabata & Nakano, 2000; Salomon, Janson & Graneli, 2003).

The release of energy-rich compounds, such as carbohydrates, by phytoplankton has been regarded as paradoxical (Passow *et al.*, 1994), as the rate of excretion increases with depletion of essential nutrients (Obernosterer & Herndl, 1995) and the associated bacteria can be efficient competitors of phytoplankton (Guerrini *et al.*, 1998). Under particular conditions, however, the rate of nutrient re-mineralisation can be sufficiently rapid to have a positive effect on the phytoplankton population (Wood & Van Valen, 1990; Passow *et al.*, 1994). In this paper we observed the population growth of three phytoplanktonic species in cultures, some of which were cultivated with a microbial community isolated from the same site from which the algae had previously been isolated, a tropical eutrophic reservoir. We also monitored bacterial growth and use of dissolved carbohydrates released by phytoplankton, using a powerful method to detect low concentrations of carbohydrate; pulse amperometric detection coupled to high performance liquid chromatography (PAD-HPLC) (Gremm & Kaplan, 1997).

Methods

Organisms and culture conditions

Phytoplanktonic species and a microbial inoculum were obtained from Barra Bonita Reservoir on the Tiete River, SP, Brazil (Latitude 22°29′S; Longitude 48°34′W). The three species chosen were numerous in the phytoplankton of the reservoir (Calijuri, 1999; Calijuri, Dos Santos & Jati, 2002). *Cryptomonas tetrapyrenoidosa* Skuja, (Cryptophyceae), *Staurastrum orbiculare* Ehrenberg Ralfs var. *orbiculare* f.*orbiculare* (Zygnematophyceae) and *Thalassiosira duostra*, Pienaar & Pieterse, (Bacillariophyceae) were isolated directly under a microscope. The cultures (maintained in the culture collection of the Department of Botany of the Federal University of São Carlos, SP, Brazil) were grown in WC medium (Guillard & Lorenzen, 1972) under 100 μmol m^{-2} s^{-1} (photosynthetically active radiation), with a 12 : 12-h dark : light cycle and at a temperature of 22 ± 1 °C. For the experiment, the concentrations of nitrate and phosphate in the WC medium were reduced by 50% (WC/2).

Axenic cultures of *C. tetrapyrenoidosa* and *S. orbiculare* were obtained by several washes with sterile medium under the microscope, followed by re-isolations. Axenic cultures of *T. duostra* were obtained by washing the cultures with Dakin solution (0.5% sodium hypochlorite w/v in sodium bicarbonate 5% sol, Merck Index, 8774 Merck & Co. Inc. Whitehouse Station, NJ, U.S.A.) (Vieira, 1983), followed by several re-isolations. Bacterial contamination tests were conducted to confirm the absence of bacteria in the axenic cultures. These were performed at the beginning, middle (8 days) and final stage (16 days) of the experimental period with WC/2 medium plus glucose and peptone at a 250 mg L^{-1} concentration (WC/2 P + G).

The microbial inoculum was obtained from the euphotic zone (0–5 m, as determined by an LI-193SA spherical quantum sensor and an LI-250 light meter LiCor, Lincoln, NE, U.S.A.) of Barra Bonita Reservoir at the same site from which the algae were taken, about a year after their isolation. The sample was aseptically filtered at low vacuum pressure (10 mmHg cm^{-1}) through GF/A 1.6-μm pore size glass-fibre filters (Whatman, Maidstone, U.K.) to remove most particles, including zoo- and phytoplankton. Microscopic observations were performed to ensure the absence of other phytoplanktonic species and heterotrophic flagellates in the microbial inoculum during the experimental period, and also the absence of bacteria in the axenic cultures. The microscopic observations were carried out every 48-h until the cultures (both axenic and co-inoculated) reach the stationary growth phase, as described in the next section.

Phytoplankton/bacteria association experiments

A 4-L carboy filled with 2.5 L of WC/2 medium was inoculated with a 200 mL-inoculum (in the exponential growth phase, approximately 10^{-6} cell mL^{-1}) of each alga and was cultivated in duplicate as above

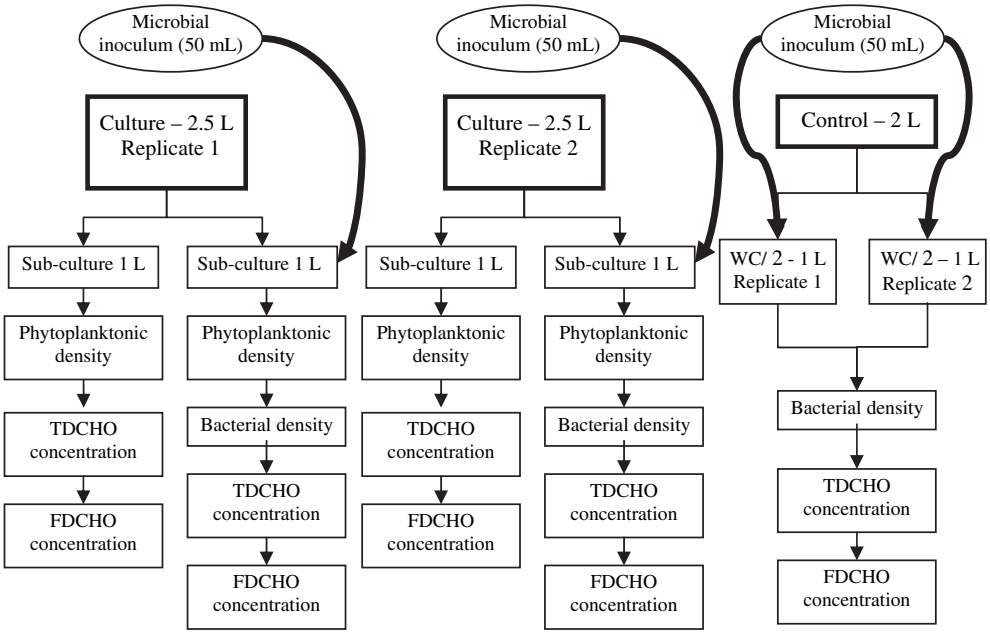

Fig. 1 Experimental design showing elaboration of the controls and the experimental cultures (sub-cultures) of each phytoplanktonic species and comparative analysis between axenic and microbial co-inoculated cultures. Phytoplanktonic density was determined by direct counts under the microscope; bacterial density was determined by counts under an epifluorescence microscope after staining bacteria with DAPI; the concentrations of total and free dissolved carbohydrates (TDCHO and FDCHO) were determined by HPLC-PAD.

(Fig. 1). After 5 days, each culture replicate was divided into two sub-cultures of 1 L each, which were placed aseptically in 2-L Erlenmeyer flasks (a total of 12 flasks). One of the l-L sub-cultures of each replicate was inoculated with 50 mL of the microbial inoculum described previously (Fig. 1). A control flask without algae was prepared in duplicate by inoculating the microbial community in the WC/2 medium and it was maintained during the whole experiment (Fig. 1). Samples were collected immediately after microbial inoculation and, afterwards, every 48-h until the cultures reach the stationary growth phase. A 10-mL aliquot was fixed with Lugol to determine algal population density and 10 mL were fixed with 4% formalin to determine bacterial growth. A 15-mL aliquot was filtered through 0.45-μm pore acetate membranes (Millipore, Dublin, CA, U.S.A.) to determine dissolved carbohydrate concentration. Algal population density was monitored by direct count under the microscope. *Cryptomonas tetrapyrenoidosa* and *T. duostra* were counted using a Fuchs-Rosenthal haemocytometer chamber, while *S. orbiculare* was counted using a Palmer-Maloney 0.1 mL chamber. The absence of other phytoplankters and flagellates during the experiment was confirmed through microscopic observations of the same samples used for the determination of algal population density, but before fixation. This sample was also used to confirm the absence of bacteria in the axenic cultures, both by microscopic observation and WC P + G contamination test. Bacterial growth was evaluated by direct counts on an epifluorescent microscope with UV and a light source (Zeiss Axioplan 2, Jena, Germany) after staining the cells with 4′6′-diamidino-2-fenilindol (DAPI) (Porter & Feig, 1980).

The dissolved carbohydrates, analysed by a PAD-HPLC, were divided into two fractions before analysis: total dissolved carbohydrates (TDCHO) and free dissolved carbohydrates (FDCHO). Combined dissolved carbohydrates (CDCHO) were also obtained by subtracting TDCHO and FDCHO. Bacterial use of both CDCHO and FDCHO was calculated by subtracting the carbohydrate concentration detected in the axenic culture from that in the microbial co-inoculated culture. The FDCHO samples were injected directly into the PAD-HPLC, while TDCHO were hydrolysed (Gremm & Kaplan, 1997) prior to injection.

The PAD-HPLC analyses were performed on a Dionex DX500 (Sunnyvale, CA, U.S.A.) device consisting of a PEEK GP40 gradient pump module, an ED40 electrochemical detector, LC5 manual injector

with a Rheodyne 9125 valve and a 25 μL peek sample loop. The ED40 detector was equipped with an amperometric flow cell, a gold working electrode, and an Ag/AgCl reference electrode. A PA-10 (Dionex) anion-exchange analytical column (4 × 250 mm), fitted with a corresponding guard-column (4 × 50 mm), was used to separate the monosaccharides. The eluent used for separation was 18 mM NaOH; while for column recuperation it was 200 mM NaOH at a flow rate of 1 mL min^{-1} (Gremm & Kaplan, 1997). To remove salts from the culture media, all samples (FDCHO and TDCHO) were treated with Bio-Rad ion exchange resin (AG2X8 – anion exchange and AG50W – cation exchange). Mannose and xylose, were detected in a single peak including mannose and/or xylose (man/xyl). This is due to their not having been well separated by the PA-10 column, as reported elsewhere (Gremm & Kaplan, 1997), the PAD-HPLC analysis was carried out without a postcolumn system. In addition, the uronic acids and amino sugars, which were <10% of the EPS, were not measured by PAD-HPLC because of retention by the desalting columns.

Results

Fig. 2 shows phytoplanktonic growth in axenic and microbial co-inoculated cultures of *C. tetrapyrenoidosa*, *S. orbiculare* and *T. duostra*. There was no difference in the growth of *C. tetrapyrenoidosa* (Fig. 2a) or *S. orbiculare* (Fig. 2b), with or without concomitant bacterial growth, while *T. duostra* achieved a greater biomass in the microbial co-inoculated culture (Fig. 2c). The numbers of bacteria increased in all phytoplankton cultures, and carbohydrate utilisation was confirmed statistically for all species (Fig. 3; Tables 1 and 2), indicating the significance of even small concentrations of these compounds as substrates for bacterial populations. Bacterial growth in the controls, in which no extracellular carbohydrates were detected by HPLC-PAD, was much lower than in the phytoplanktonic cultures.

Bacterial growth in the *C. tetrapyrenoidosa* (Fig. 3a) culture was rapid in the first 3 days of incubation ($0.063–4.5 \times 10^{-6}$ mL^{-1}), though population density decreased between 3 and 9 days of incubation. Use by bacteria of FDCHO released by *C. tetrapyrenoidosa* was observed from the beginning of the experiment and increased continuously throughout. Use of CDCHO

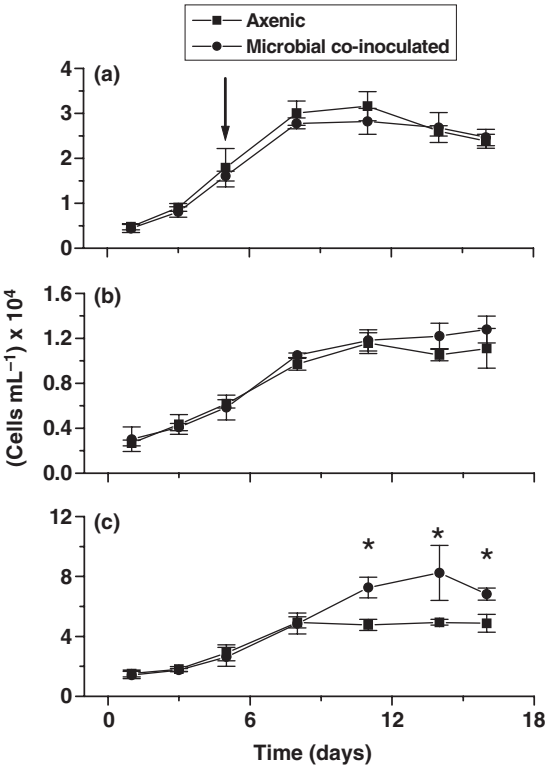

Fig. 2 Growth in batch cultures of *Cryptomonas tetrapyrenoidosa* (a), *Staurastrum orbiculare* (b) and *Thalassiosira duostra* (c) in axenic and microbial co-inoculated cultures. The arrow represents the microbial inoculation. The asterisks indicate significant differences between axenic and microbial co-inoculated cultures (Student's *t*-test, $P < 0.05$). Points are mean (±SD, $n = 2$).

was observed only 6 days after incubation (Fig. 3a). When FDCHO concentration in the microbial co-inoculated culture reached a minimum (Table 2), bacterial density decreased, with growth restarting only after a significant increase in CDCHO concentration (Table 1).

In *S. orbiculare*, population growth was distinct from that in *C. tetrapyrenoidosa*. Growth started at the beginning of the experiment ($0.07–1.3 \times 10^{-6}$ bacteria mL^{-1}) but stopped after 6 days. The use of FDCHO was marked from the outset of the experiment until day 6, when FDCHO was exhausted in the microbial co-inoculated culture. The percentage use decreased after 9 days. The use of CDCHO began only after day 6 and increased thereafter until the end of the experiment (Tables 1 and 2; Fig. 3b).

A third pattern of bacterial growth was observed in *T. duostra*, in which numbers increased steadily throughout the experiment, starting with 0.07 ×

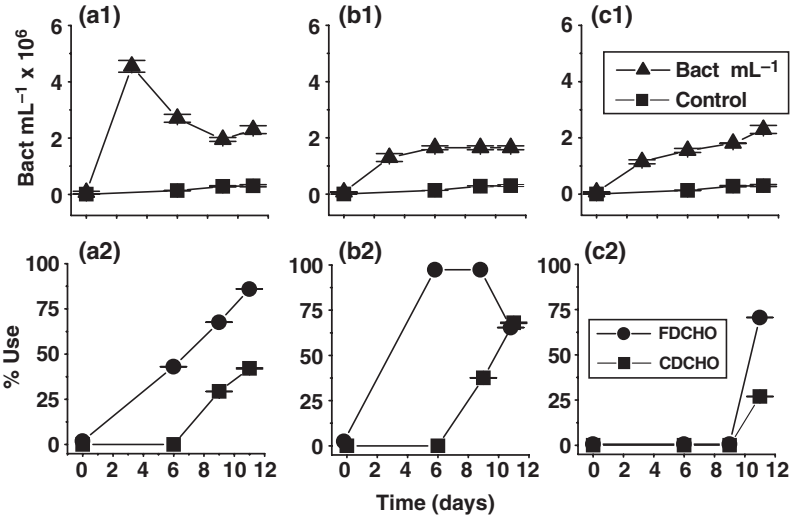

Fig. 3 Bacterial density (1) and percentage of use (2) of both combined (CDCHO) and free dissolved carbohydrate (FDCHO) in the microbial co-inoculated culture of (a) *Cryptomonas tetrapyrenoidosa*, (b) *Staurastrum orbiculare* and (c) *Thalassiosira duostra* during the experimental period. Points are mean (±SD, $n = 2$).

Table 1 Combined dissolved carbohydrate (CDCHO) concentrations in axenic and microbial co-inoculated cultures (mg L^{-1}) of (a) *Cryptomonas tetrapyrenoidosa*, (b) *Staurastrum orbiculare* and (c) *Thalassiosira duostra*.

	Initial	6 days	9 days	11 days
(a)				
Axenic	0.63 ± 0.11	0.74 ± 0.08	1.60 ± 0.12	1.88 ± 0.41
Co-inoculated	0.66 ± 0.16	0.90 ± 0.10	1.13 ± 0.17	1.09 ± 0.10
P-value	0.8025	0.1162	0.0203*	0.0340*
Use	0	0	0.47 ± 0.05	0.79 ± 0.31
(b)				
Axenic	0.370 ± 0.01	0.448 ± 0.004	0.630 ± 0.04	0.742 ± 0.09
Co-inoculated	0.368 ± 0.02	0.434 ± 0.03	0.393 ± 0.04	0.237 ± 0.02
P-value	0.8961	0.5013	0.0031*	0.0007*
Use	0	0	0.237 ± 0.04	0.505 ± 0.07
(c)				
Axenic	0.41 ± 0.09	0.548 ± 0.037	0.375 ± 0.06	0.725 ± 0.026
Co-inoculated	0.55 ± 0.05	0.694 ± 0.042	0.464 ± 0.02	0.529 ± 0.084
P-value	0.0803	0.2339	0.0745	0.0184*
Use	0	0	0	0.196 ± 0.058

The *P*-value refers to the comparison (Student's *t*-test) between concentrations of CDCHO in the axenic and microbial co-inoculated cultures. Use of CDCHO was calculated by subtracting CDCHO concentration in the axenic culture from that of the microbial co-inoculated culture.
*Indicates the significant difference.

10^{-6} mL^{-1} and reaching 2.3×10^{-6} mL^{-1} after 11 days of incubation (Fig. 3c). The use of both CDCHO and FDCHO commenced after only 9 days of incubation (Tables 1 and 2). However, at no time did the concentrations of most hydrophobic monosaccharides (galactose and the deoxysugars fucose and rhamnose) of the CDCHO released by *T. duostra* decrease in the microbial co-inoculated culture. Bacteria did not use them and probably did not produce them (thus potentially masking their use), as no extracellular carbohydrates were detected in the controls. Mannose/xylose and glucose, whose use began on day 6, were probably the principal sources of carbohydrate for bacterial growth (Fig. 4).

Discussion

Previous studies of phytoplankton/bacteria associations and their specificity (Bell & Mitchell, 1972; Bell & Sakshaug, 1980; Fallowfield & Daft, 1988; Salomon *et al.*, 2003) have not yet determined whether such associations are commensal or mutualistic. Our results confirmed the use of both CDCHO and FDCHO by bacteria (Agis *et al.*, 1998; Yallop, Paterson

	Initial	6 days	9 days	11 days
(a)				
Axenic	0.014 ± 0.003	0.017 ± 0.002	0.035 ± 0.009	0.044 ± 0.012
Co-inoculated	0.019 ± 0.004	0.010 ± 0.002	0.012 ± 0.001	0.007 ± 0.0005
P-value	0.0603	0.0191*	0.0122*	0.0071*
Use	0	0.007 ± 0.002	0.023 ± 0.008	0.037 ± 0.009
(b)				
Axenic	0.017 ± 0.001	0.014 ± 0.001	0.019 ± 0.004	0.030 ± 0.001
Co-inoculated	0.023 ± 0.003	0	0	0.011 ± 0.001
P-value	0.0550	0*	0*	0.0001*
Use	0	0.014 ± 0	0.019 ± 0	0.019 ± 0.001
(c)				
Axenic	0.007 ± 0.001	0.018 ± 0.002	0.014 ± 0.0005	0.020 ± 0.001
Co-inoculated	0.007 ± 0.001	0.015 ± 0.004	0.014 ± 0.0005	0.006 ± 0.0005
P-value	0.999	0.2696	0.999	0.0002*
Use	0	0	0	0.014 ± 0.0005

Table 2 Free dissolved carbohydrate (FDCHO) concentrations in the axenic and microbial co-inoculated cultures (mg L^{-1}) of (a) *Cryptomonas tetrapyrenoidosa*, (b) *Staurastrum orbiculare* and (c) *Thalassiosira duostra*

The *P*-value refers to the comparison (Student's *t*-test) between concentrations of FDCHO in the axenic and microbial co-inoculated cultures. Use of FDCHO was calculated by subtracting FDCHO concentration in the axenic culture from that of the microbial co-inoculated culture.
*Indicates the significant difference.

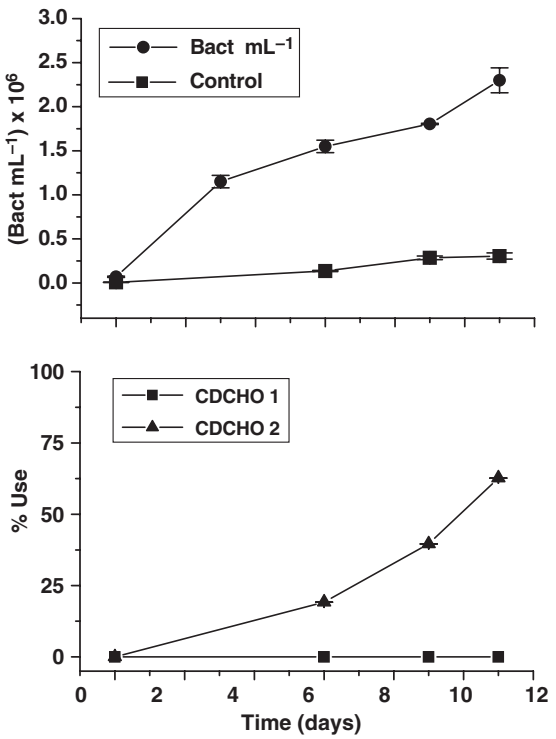

Fig. 4 Percentage of bacterial use of combined dissolved carbohydrate (CDCHO) from group 1 (fucose, rhamnose and galactose) and group 2 (glucose and mannose/xylose), correlated with bacterial growth in the microbial-microbial co-inoculated culture of *Thalassiosira duostra* during the experimental period. Points are mean (±SD, $n = 2$).

& Wellsbury, 2000; Rosenstock & Simon, 2003). However, different growth patterns and carbohydrate use were observed for each phytoplanktonic species, suggesting that substrate composition could influence bacterial behaviour or select different bacterial populations. Previous papers have shown great variation in the extracellular carbohydrates produced by *C. tetrapyrenoidosa*, *S. orbiculare* and *T. duostra* (Giroldo *et al.*, 2003; Giroldo, Vieira & Paulsen, 2005a,b; Giroldo & Vieira, 2005). Future studies will be carried out to investigate the association's specificity.

Our results also showed that phytoplanktonic extracellular organic compounds, including carbohydrates, supported dense bacterial populations even at very low concentrations, as suggested by others (Wood & Van Valen, 1990). Data in the literature are contradictory on the significance of high molecular weight (HMW) carbohydrates, whereas low molecular weight carbohydrates are generally considered more important in promoting rapid bacterial growth (Lancelot, 1984; Sundh, 1992), The use of HMW carbohydrates, however, both as a mechanical support and a carbon source, has been demonstrated by recent studies (Giroldo *et al.*, 2003, 2005a,b; Colombo *et al.*, 2004). In addition, Amon & Benner (1996) have proposed a new concept of bioreactivity of HMW organic compounds in natural waters based on demonstrations that these compounds are the major

substrates for bacterial metabolism and, in accordance with their size-reactivity continuum model, larger organic matter molecules are considered more reactive than smaller molecules. However, data obtained through experiments on microbial degradation of phytoplanktonic polysaccharides are insufficient to identify patterns in their degradation kinetics and their use as a carbon source by microbial communities.

Our results did show that both FDCHO and CDCHO were significant for bacterial growth. In the microbial co-inoculated cultures of *S. orbiculare*, use of FDCHO decreased after 9 days whereas, in the same period, use of CDCHO was maximised and probably supported the bacteria. Furthermore, in the *C. tetrapyrenoidosa* microbial co-inoculated culture, use of CDCHO was significant when the concentration of FDCHO was far too low to support the bacterial population. In addition, the *C. tetrapyrenoidosa* co-inoculated culture showed a decrease in bacterial numbers after 3 days and, as we inoculated a bacterial community from Barra Bonita reservoir, it was expected that there would be several populations with different requirements and growth patterns. It was also expected that the abundance of such bacterial populations would vary during the experiment. When we showed a bacterial density decrease, concomitant with the use of DCHO (Fig. 3a), it indicated that the principal fast-growing bacterial populations were decreasing and other, slower growing bacterial populations were probably increasing using algal DCHO. Such behaviour is supported by the slight increase (t- test $P = 0.04$) in bacterial density between days 9 and 11 (Fig. 3), presumably because of an increase in bacteria responsible for use of both FDCHO and CDCHO.

In the *T. duostra* microbial co-inoculated culture, FDCHO use was significant only after 9 days of incubation and the hydrophobic components of CDCHO were not used by the bacterial community at any time during the experiment. On the other hand, the hydrophilic components of CDCHO were used from day 6, exactly when the microbial co-inoculated culture showed an increase in the *T. duostra* population density compared with the axenic cultures. As the total concentration of CDCHO showed no differences until day 9, it showed that other organic compounds, such as aminoacids, organic acids and non-detected sugars (e.g. uronic acids and amino sugars), must have had a significant role in promoting bacterial growth, besides CDCHO hydrophilic components and FDCHO. Future studies should focus on the significance of these compounds on the growth of bacteria associated with *T. duostra*. Although other organic compounds were not considered here and the bacteria probably use these compounds, the significance of both FDCHO and CDCHO for bacterial growth dynamics was clearly demonstrated.

Cryptomonas tetrapyrenoidosa and *S. orbiculare* showed no change in growth when cultivated with bacterial, as was also observed by Salomon *et al.* (2003) for *Nodularia spumigena* Mertens. *Thalassiosira duostra* developed a greater biomass when cultivated with bacteria. In addition, use of CDCHO by *T. duostra* showed a specific pattern, characterised by an apparent absence of degradation of the deoxysugars (fucose and rhamnose) during the experiment. This result can be correlated with the selective pattern of carbohydrate degradation observed in *T. duostra*, which has also been noted previously (Aluwihare & Repeta, 1999; Giroldo *et al.*, 2003; Giroldo & Vieira, 2005). Giroldo *et al.* (2003) described the bacterial degradation of the exopolysaccharide of*Thalassiosira* sp. and found the same selective pattern of behaviour.

The apparent lack of fucose, rhamnose and galactose degradation was probably not because of the release of these carbohydrates by bacteria, as no detectable release was found in the controls. However, as the bacterial growth profile found in the controls was very different from that in the cultures, there might have been different bacterial populations in the cultures. Thus, we could not completely eliminate the possibility of bacterial production of a deoxysugar-rich carbohydrate, which would mask the degradation of such compounds in the cultures. However, independent of the deoxysugar origin, these sugars have been reported to have hydrophobic properties. As these properties became more pronounced throughout the incubation period, the carbohydrate pool also became more hydrophobic (Giroldo *et al.*, 2003) and, therefore, more susceptible to aggregation, as the enhancement of the hydrophobic properties of TDCHO increases the tendency for aggregation in the medium (Wustman, Gretz & Hoagland, 1997).

In conclusion, we observed bacterial growth concomitant with phytoplanktonic growth in the three species studied. Furthermore, bacteria used algal-

derived carbohydrates (both FDCHO and CDCHO) even at concentrations <1 mg L^{-1}. *Thalassiosira duostra* was positively influenced by concomitant bacterial growth, and the pattern of carbohydrate use by bacteria increased the percentage of deoxy sugars in the extracellular medium, making it more hydrophobic and probably more susceptible to aggregation.

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