JOURNAL OF PLANKTON RESEARCH | VOLUME 27 | NUMBER 7 | PAGES 695–705 | 2005

Polymeric and free sugars released by three phytoplanktonic species from a freshwater tropical eutrophic reservoir

DANILO GIROLDO[1]* AND ARMANDO AUGUSTO HENRIQUES VIEIRA[2]

[1]DEPARTAMENTO DE CIENCIAS MORFOBIOLOGICAS, FUNDACAO UNIVERSIDADE FEDERAL DO RIO GRANDE, AV ITALIA KM 8, RIO GRANDE, RS 96201-900, BRAZIL AND [2]DEPARTAMENTO DE BOTÂNICA, UNIVERSIDADE FEDERAL DE SÃO CARLOS, VIA WASHINGTON LUIS KM 235, SÃO CARLOS, SP 13565-905, BRAZIL

*CORRESPONDING AUTHOR: dmbgirol@furg.br

Received February 18, 2005; accepted in principle May 19, 2005; accepted for publication July 1, 2005; published online July 26, 2005

Communicating editor: K.J. Flynn

Several studies have focused on the release of carbohydrates by phytoplankton because of the ecological significance of such compounds. This process increases the supply of carbon to the heterotrophic community, enhancing the phytoplankton/bacteria associations. In this article, we report investigations on the carbohydrate release, both polymeric and free sugars, in axenic batch cultures of three tropical freshwater phytoplanktonic species from different taxonomic positions: Cryptomonas tetrapyrenoidosa (Cryptophyceae), Staurastrum orbiculare (Zygnematophyceae) and Thalassiosira sp. (Bacillariophyceae). The total carbohydrate release rate was increased in the stationary growth phase in all the species under study. Most of the carbohydrates released by the three species were present in the polymeric form, although both polymeric and free carbohydrates could supply carbon enough to support bacterioplanktonic populations, according to rates of consumption found on literature. The composition of the carbohydrates differed significantly from one species to another, indicating that carbohydrate release might be a species-specific process. We also observed that the contributions of some components from polymeric sugars, such as fucose, rhamnose and arabinose increased with the advancing age of the cultures.

INTRODUCTION

Although Bjørnsen (Bjørnsen, 1988) postulated that the excretion of dissolved organic matter by phytoplankton would be a passive rather than an active process, the release of extracellular polysaccharides (EPS), at least, must be an active process involving Golgi vesicles (Domozych and Domozych, 1993), with the release rate depending on the physiological and environmental conditions (Vieira and Myklestad, 1986; Giroldo and Vieira, 2002). Under nitrogen and/or phosphorus limitation, phytoplanktonic populations are assumed to decrease their growth, inhibiting or slowing down their cytokinesis while photosynthesis is still active. It is also assumed that the combination of nutrient limitation and saturating light produces an excess of photoassimilated carbon (Fogg, 1983). The allocation of this excess or the reallocation of internal carbon stores increases the production of carbohydrates (Myklestad, 1995) and other carbon-rich molecules such as lipids and organic acids (Mayzaud *et al.*, 1989; Biddanda and Benner, 1997).

Wood and Van Valen (Wood and Van Valen, 1990) extended the hypothesis of carbon excess, postulating an adaptive function for carbohydrates released under a specific set of conditions. According to this function, in a transient habitat where nutrient availability would be temporally uncoupled from periods of excessive light, the release of carbohydrates by phytoplankton would be increased and would support carbon-limited bacterial populations. The release of carbohydrates in such conditions would be a mechanism to protect the photosynthetic machinery, and the bacterial growth would return essential nutrients to the nutrient-limited phytoplankton, in addition to support a bacterivorous population. Although the authors mentioned the lack of studies on carbohydrate release by phytoplanktonic species in axenic cultures, more recent literature offers more data (Paulsen *et al.*, 1992; Paulsen and Vieira, 1994; Lombardi *et al.*, 1998). However, the characterization of carbohydrates released by phytoplanktonic species of distinct taxonomic position isolated from different sites is still rare.

doi:10.1093/plankt/fbi043, available online at www.plankt.oxfordjournals.org

Wood and Van Valen's hypothesis is based, essentially, on the release of simple carbohydrates, but many authors have reported the significance of polymeric carbohydrates, which have been identified as the main exuded material (Lancelot, 1984; Myklestad, 1995; Biddanda and Benner, 1997). Also, high molecular weight organic compounds have been considered more reactive than low molecular weight ones to the bacterial community (Amon and Benner, 1996). The release of free and polymeric carbohydrates may play an important role in the specific phytoplankton/bacteria associations mentioned by several authors but which remain little studied (Bell and Mitchell, 1972; Bell and Sakshaug, 1980). Bacteria are believed to assimilate free carbohydrates (Lancelot, 1984), while polysaccharides would be used either as a carbon source (Freire-Nordi and Vieira, 1996, 1998) or as a mechanical support for the associated bacteria. The area under the influence of this association is known as phycosphere (Bell and Mitchell, 1972); however, the role of carbohydrates hasnot been completely clarified, nor whether these phytoplankton/bacteria associations would be commensal or the two organisms would take advantages of the relationship.

The aim of this work was to perform a qualitative and quantitative analysis of the free and polymeric carbohydrates released under optimum conditions of growth by axenic cultures. Three tropical freshwater phytoplanktonic species of different taxonomic positions were investigated: a Bacillariophyceae, a Cryptophyceae and a Zygnematophyceae. Based on data from the literature, we also calculated whether both forms of carbohydrates could supply enough carbon to support a bacterial population associated with the algal cells.

Table I: Nutrient concentration (mg L^{-1}) in the WC medium (Guillard and Lorenzen, 1972) and in the Barra Bonita reservoir (Calijuri, 1999 and Soares, 2003)

Nutrient	WC medium	Barra Bonita reservoir
$CaCl_2$	36.76	20
$MgSO_4$	36.97	30
$NaHCO_3$	12.60	42.9
$NaNO_3$	85.01	2.45
K_2HPO_4	8.71	1.0
$NaSiO_3$	28.41	11.43
Na_2EDTA	0.43	*
$FeCl_3 \cdot 6H_2O$	0.31	*
$CuSO_4 \cdot 5H_2O$	0.001	*
$ZnSO_4 \cdot 7H_2O$	0.002	*
$CoCl_2 \cdot 6H_2O$	0.001	*
$MnCl_2 \cdot 4H_2O$	0.018	*
$Na_2MoO_4 \cdot 2H_2O$	0.0006	*
H_3BO_3	0.1	*
Thiamin HCl	0.01	*
Biotin	0.00005	*

The asterisk-marked nutrients have no available information for Barra Bonita reservoir in literature data.

METHOD

Organisms and culture conditions

The phytoplanktonic species were isolated from the Barra Bonita Reservoir on the Tiete river, SP, Brazil (22°29́ S and 48°34́ W). We isolated directly on the microscope *Cryptomonas tetrapyrenoidosa* Skuja, (Cryptophyceae), *Staurastrum orbiculare* Ehrenberg Ralfs var. *orbiculare f. orbiculare* (Zygnematophyceae) and *Thalassiosira* sp. (probably *Thalassiosira duostra*) (Bacillariophyceae), based on the importance of these species in the phytoplanktonic community. The cultures were grown in WC medium (Guillard and Lorenzen, 1972), summarized in Table I, under 100 μmol m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12 h dark : light cycle and a temperature of 22 ± 1°C, in the microalgal culture collection of the Department of Botany at the Federal University of Sao Carlos, SP, Brazil. Axenic cultures of *C. tetrapyrenoidosa* and *S. orbiculare* were obtained by several washes with sterile medium on the microscope, followed by re-isolations. Axenic cultures of *Thalassiosira* sp. were obtained by washing the cultures with Dakin solution (Vieira, 1983), followed by several re-isolations. Bacterial contamination tests were conducted regularly with WC medium plus glucose and peptone at the concentration of 250 mg L^{-1} each.

Phytoplanktonic growth experiments

A 9-L flask (Pirex) filled with 7 L of WC medium was inoculated with a 300 mL inoculum (exponential growth phase, approximately 10^6 cell mL^{-1}) for each algae and cultivated under the above described conditions. Samples were collected at 24 h intervals in the first 7 days and every 48 h during the remainder of the period, until the cultures reach the stationary growth phase. Five milliliters were fixed with lugol to determine the cell density and 15 mL were filtered through 0.45 μm pore acetate membranes (Millipore) to determine the total dissolved carbohydrate concentration. The cell density (4 replicates) was monitored by direct count under the microscope. *Cryptomonas tetrapyrenoidosa* and *Thalassiosira*

sp. were counted in a Fuchs-Rosenthal hemocytometer chamber, while *S. orbiculare* was counted in a Palmer-Malloney 0.1-mL chamber. The total dissolved carbohydrates were monitored by the phenol-sulphuric method (Dubois *et al.*, 1956) in triplicate and the amounts were determined using a glucose standard curve fitted with several concentrations in the sample concentration range. The carbohydrates were also analyzed by gel filtration column chromatography and high performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC), whose details are presented in the next section.

Carbohydrate characterization

Every 7 days, 100-mL samples were collected to characterize the carbohydrates. The samples were first concentrated three times in a rotary evaporator at 40°C. Gel filtration column chromatography was performed to observe the molecular weight distribution of the carbohydrates released by the three species. The running conditions were bed dimensions = 1.5 × 50 cm, flow rate = 1mL 7min^{-1}, eluent = distilled water + 2% butanol, load volume = 5% of the column's total volume (6 mL) and running at environmental temperature. The column was calibrated using standard dextrans of 10^4 and 2×10^6 D. The gel used was P-10 Bio Rad, with an exclusion limit between 2.5×10^2 and 2×10^4 D, to observe the presence of low molecular weight carbohydrates. Carbohydrates were fractionated in a Pharmacia Frac 200 device and detected in the 5 mL fractions by the phenol-sulphuric method (Dubois *et al.*, 1956). The fractions with significant amounts of carbohydrates were pooled and analyzed, after hydrolysis (Gremm and Kaplan, 1997), by PAD–HPLC. The carbohydrates located in the final fractions were not hydrolyzed to detect the free carbohydrates. The PAD–HPLC analyses were performed on a Dionex DX500 device consisting of a PEEK GP40 gradient pump module, an ED40 electrochemical detector and an LC5 manual injector with a Rheodyne 9125 valve with 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 the corresponding guard-column (4 × 50 mm), was used to separate the monosaccharides. The eluent used for the separation was 18 mM NaOH, with 200mM NaOH for column recuperation, at a flow rate of 1 mL min^{-1} (Gremm and Kaplan, 1997). All the samples (free and polymeric sugars) were desalted on Bio Rad ionic exchange resin (AG2X8–anion exchange and AG50W–cation exchange) to remove salts from the culture media and hydrolysis.

Organic carbon analysis

Samples (50 mL) were collected at the stationary, initial and final exponential growth phase in order to evaluate the cellular and dissolved organic carbon increase (four replicates) and their relation to the carbohydrate release. Total organic carbon was determined with a carbon analyzer (TOC-VCPH Shimadzu) analyzing both cells and culture media homogenized in a stirring plate (Fisatom 752, Sao Paulo, Brazil). Dissolved organic carbon (DOC) was determined only for the culture media, after filtration through prewashed (200 mL of distilled water) 0.45-μm pore acetate membranes (Millipore). Tests were carried out to certify that the samples were not contaminated by the membranes. Particulate organic carbon (POC) was obtained with the subtraction of total and dissolved organic carbon.

RESULTS

Figure 1A depicts the growth of *C. tetrapyrenoidosa*. The growth rate was 0.15 divisions per day, calculated using the equation described by Fogg (Fogg, 1975). The stationary growth phase was reached after ~20 days and no significant decrease of cell density was observed for ~50 days [analysis of variance (ANOVA0 $P = .10$]. *Staurastrum orbiculare* showed the slowest growth rate, i.e. 0.09 division per day (Fig. 1B). The stationary growth phase was reached after 46 days and no significant decrease in cell density was observed until about the 60th day (ANOVA $P = 0.21$). *Thalassiosira* sp. displayed the fastest growth rate: 0.59

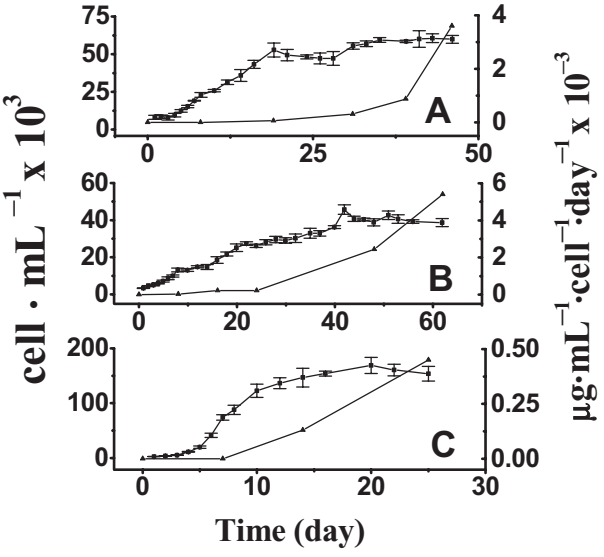

Fig. 1. Cellular density (right axis/squares) and rate of total dissolved carbohydrates release (left axis/triangles) as a function of time of (**A**) *Cryptomonas tetrapyrenoidosa*, (**B**) *Staurastrum orbiculare* (**C**) *Thalassiosira* sp.

divisions per day (Fig. 1C). The growth rate slowed down considerably between the 12th and the 20th days, dropping to 0.04 divisions per day and evidencing a transition phase. Moreover, the cell density showed no significant variations between the 21st and 25th days (ANOVA $P = 0.20$).

The rates of total dissolved carbohydrate release were also dissimilar with *S. orbiculare* releasing more carbohydrates than the other two species (Fig. 1A), i.e. approximately 6×10^{-3} μg cell^{-1} day^{-1} by the end of the experiment. *Cryptomonas tetrapyrenoidosa* (Fig. 1B) released approximately 4×10^{-3} μg cell^{-1} day^{-1} after 50 days and *Thalassiosira* sp. (Fig. 1C) released approximately 0.5×10^{-3} μg cell^{-1} day^{-1} after 25 days. Overall, the total dissolved carbohydrate release rate was clearly higher in the stationary growth phase of the three species (Fig. 1A, B and C). Table II summarizes, *C. tetrapyrenoidosa*, *S. orbiculare* and *Thalassiosira* sp., the concentrations per cell of POC, DOC, extracellular carbohydrates (CHO) and the relation between CHO and POC, as well as CHO and DOC, at the beginning and end of exponential growth phase, in addition to that at stationary growth phase. POC cell^{-1} increased significantly only in *Thalassiosira* sp. cultures until the end of the stationary [ANOVA $P = .011$, Tuckey $7 < (14 = 25)$]. DOC cell^{-1} increased significantly in *C. tetrapyrenoidosa* (ANOVA $P = .011$, Tuckey $7 < 20 < 40$) and *Thalassiosira* sp. cultures [ANOVA $P = .005$, Tuckey $(7 = 14) < 25$], especially at the stationary growth phase. The relation between POC and CHO cell^{-1} increased significantly in all species especially at the stationary growth phase, *C. tetrapyrenoidosa* [ANOVA $P = .009$, Tuckey $(7 = 20) < 40$], *S. orbiculare* [ANOVA $P = .008$, Tuckey $(7 = 40) < 60$] and *Thalassiosira* sp. [ANOVA $P = .002$, Tuckey $(7 = 14) < 25$]. The relation between DOC and CHO cell^{-1} was not significantly increased in any cultivated species.

Figure 2 shows the molecular weight distribution of the carbohydrates released by *C. tetrapyrenoidosa*, *S. orbiculare* and *Thalassiosira* sp. during their growth, using P-10 gel filtration column chromatography. Polymeric carbohydrates comprised most of the sugars released by the three species, as confirmed by PAD–HPLC analysis. The high molecular weight (HMW) carbohydrates ranged from 89.8 to 100%, 94.9 to 99 % and 88.9 to 95.8% of the total carbohydrates released by *C. tetrapyrenoidosa*, *S. orbiculare* and *Thalassiosira* sp., respectively, while the free monosaccharides ranged from 0 to 10.2%, 1 to 5.1% and 4.2 to 11.1 %, respectively (Tables III, IV and V, '% 1'). The free carbohydrates released per cell of *C. tetrapyrenoidosa*, *Thalassiosira* sp. and *S. orbiculare* represented 0, 0.004 and 0.02 mg L^{-1} of the total carbohydrates and the polymeric carbohydrates 0.34, 0.031 and 1.08 mg L^{-1} of the total carbohydrate per cell upon conclusion of the experiments, respectively.

C tetrapyrenoidosa released two groups of polymeric carbohydrates with different molecular weights. The HMW carbohydrates, approximately 2×10^6 D (Ve Vt^{-1} = 0–0.3), were composed of fucose, rhamnose, galactose, glucose and mannose and/or xylose (man/xyl), as shown in Fig. 3 and Table III. The PA-10 column did not separate mannose and xylose because the analyses were performed without the post column system. The intermediate molecular weight (IMW) carbohydrates, between 2×10^6 D and 10^4

Table II Concentration (mg L^{-1} cell^{-1}) of particulate organic carbon (POC), dissolved organic carbon (DOC), extracellular carbohydrate (CHO), besides the relation between CHO/POC and CHO/DOC at the beginning of exponential, end of exponential and stationary growth phase

Samples	POC	DOC	CHO	CHO/POC	CHO/DOC
Cryptomonas tetrapyrenoidosa					
7 days	1.5 ± 0.07	0.26 ± 0.007	0.14 ± 0.014	0.09 ± 0.005	0.53 ± 0.07
20 days	1.5 ± 0.14	0.33 ± 0.021	0.11 ± 0.007	0.07 ± 0.003	0.34 ± 0.001
46 days	1.1 ± 0.14	0.51 ± 0.014	0.34 ± 0.014	0.31 ± 0.05	0.66 ± 0.05
Staurastrum orbiculare					
7 days	1.95 ± 0.49	0.64 ± 0.24	0.39 ± 0.03	0.21 ± 0.03	0.65 ± 0.19
40 days	2.4 ± 0.42	0.97 ± 0.04	0.79 ± 0.04	0.35 ± 0.04	0.81 ± 0.07
62 days	2.2 ± 0.42	1.05 ± 0.07	1.1 ± 0.14	0.50 ± 0.03	0.95 ± 0.06
Thalassiosira sp.					
7 days	0.024 ± 0.0028	0.010 ± 0.0007	0.007 ± 0.0014	0.29 ± 0.028	0.66 ± 0.091
14 days	0.064 ± 0.0099	0.020 ± 0.0035	0.013 ± 0.0021	0.21 ± 0.007	0.65 ± 0.007
25 days	0.068 ± 0.0049	0.044 ± 0.0049	0.035 ± 0.0042	0.51 ± 0.028	0.78 ± 0.007

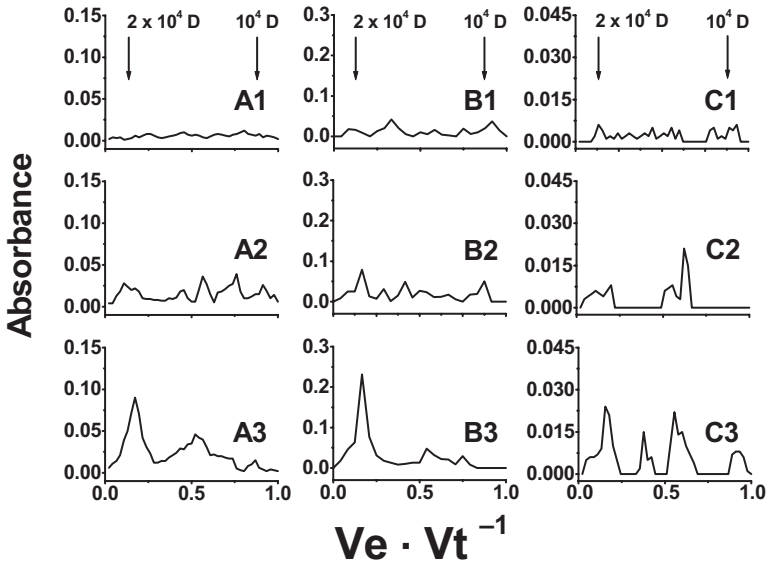

Fig. 2. Gel filtration column chromatography Bio Rad™ P-10 of the carbohydrates released during the growth by (**A**) *Cryptomonas tetrapyrenoidosa*, (**B**) *Staurastrum orbiculare* and (**C**) *Thalassiosira* sp. (**1**) Initial exponential phase, (**2**) final exponential phase and (**3**) stationary growth phase.

D (Ve Vt^{-1} = 0.3–0.7), were composed of the same monosaccharides as the HMW carbohydrates, however, in different proportions (Fig. 3, Table III '% 2'). Only glucose was found in the free carbohydrates (Ve Vt^{-1} = 0.8–1.0) in the first 7 days, accounting for 10.2% of the total carbohydrates released in the first week (Fig. 3, Table III).

Staurastrum orbiculare released only one group of polymeric carbohydrates. The HMW carbohydrates, approximately 2 × 10^6 D (Ve Vt^{-1} = 0–0.4), comprised fucose, rhamnose, arabinose, galactose, glucose and man/xyl (Fig. 4, Table IV). Although the components of the HMW carbohydrates released by *S. orbiculare* had been almost the same of *C. tetrapyrenoidosa*, the proportion of each one was quite different in all of the growth phases, besides the presence of arabinose in *S. orbiculare* (Table IV '% 2'). *S. orbiculare* released a large variety of monosaccharides in the free carbohydrates (Fig. 4, Table IV), which contained fucose, rhamnose, arabinose, galactose, glucose and man/xyl. The composition of LMW was similar to that of the HMW carbohydrates, differing in the proportion of monosaccharides (Table IV '% 2').

Thalassiosira sp. released two groups of polymeric carbohydrates with different molecular weights. The HMW carbohydrates, approximately 2 × 10^6 D (Ve Vt^{-1} = 0–0.3), differed substantially from the other two species and consisted essentially of man/xyl with smaller amounts of fucose, rhamnose, galactose and glucose (Fig. 5, Table V). The IMW carbohydrates, between 2 × 10^6 and 10^4 D (Ve Vt^{-1} = 0.4–0.7), were released in small amounts (18.2 % of the total carbohydrate released) and their composition was similar to that of the HMW carbohydrates, differing in the proportion of monosaccharides, particularly glucose and man/xyl (Fig. 5, Table V '% 2'). The free carbohydrates, also released in small amounts (11.1% of the total carbohydrate released), consisted only of fucose and glucose (Fig. 5, Table V).

The composition of the carbohydrates released by the three species changed significantly during their growth (Tables III, IV and V '% 2'). The percentage of fucose in all the species increased during growth, while the percentage of Rhamnose also rose in *C. tetrapyrenoidosa* and *Thalassiosira* sp., as did the percentage of Arabinose in *S. orbiculare*. Uronic acids and amino sugars were not detected because they were trapped by the desalting columns, but preliminary tests revealed that these components represented less than 10% of the total carbohydrates.

DISCUSSION

Small *Cryptomonas* species and diatoms have been mentioned in the literature as fast growing species (Klaveness, 1985). On the other hand, the slower growth rate of *S. orbiculare* may be related with the cell's size. Banse (Banse, 1976) and Sheldon (Sheldon, 1984) found that growth rates decreased as cell sizes increased and *S. orbiculare* has almost double the cell volume of *C. tetrapyrenoidosa* and *Thalassiosira* sp. However, the growth rates shown here cannot be exactly the same to that of a natural population, since other parameters found in nat-

Table III: Composition of the carbohydrates released by Cryptomonas tetrapyrenoidosa during the growth evaluated by high performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC)

Sugars	Fraction	Exponential growth–initial (7 days)		Exponential growth–final (20 days)		Stationary growth (50 days)	
		% 1	% 2	% 1	% 2	% 1	% 2
Fucose	1	78.6	40.7	93.2	52.0	95.8	54.8
	2	21.4	11.5	6.8	5.4	4.2	5.6
	3	0	0	0	0	0	0
	Total	100	23.7	100	33.2	100	39.6
Rhamnose	1	0	0	72.2	9.8	76.1	16.2
	2	0	0	27.8	5.4	24.9	11.3
	3	0	0	0	0	0	0
	Total	0	0	100	8.1	100	14.7
Galactose	1	70.0	26.0	43.7	15.9	46.5	14.3
	2	30.0	11.5	56.3	29.3	53.5	38.1
	3	0	0	0	0	0	0
	Total	100	17.0	100	21.7	100	22.0
Glucose	1	22.7	18.5	24.4	7.5	26.7	2.7
	2	50.0	42.4	75.6	33.8	73.3	16.3
	3	27.3	100	0	0	0	0
	Total	100	37.3	100	18.5	100	6.9
Man/Xyl	1	30.8	14.8	41.5	12.8	47.3	11.5
	2	69.2	34.6	58.5	26.1	52.7	28.7
	3	0	0	0	0	0	0
	Total	100	22.0	100	18.5	100	16.8
Total	1	45.7	100	58.9	100	69.1	100
	2	44.1	100	41.1	100	30.9	100
	3	10.2	100	0	100	0	100
	Total	100	100	100	100	100	100

Fraction 1, high molecular weight (HMW) carbohydrates; fraction 2, intermediary molecular weight (IMW) carbohydrates and fraction 3, free carbohydrates. % 1 shows the distribution of the monosaccharides in each fraction and % 2 shows the percentage of the monosaccharides in the total carbohydrates for each fraction.

ural waters must be unlike those of the laboratory, such as pH and irradiance variations as well as interspecific competition. Moreover, WC medium had been prepared with dissimilar concentrations of nitrate and phosphate of those found in Barra Bonita reservoir (Table I), in order to demonstrate the entire growth behavior of the phytoplanktonic species, including initial, exponential and stationary growth phase. In spite of the nutrient concentration dissimilarity, such growth patterns are frequently found in the Barra Bonita reservoir where large blooms of several phytoplanktonic species start, growth and disappear over the year (Calijuri *et al.*, 2000). Although nutrient concentrations in batch cultures have usually been higher than those found in natural environments, batch cultures are an acknowledged method to simulate physiological effects caused by the ageing and growth of phytoplanktonic populations.

Our results showed marked differences in the carbohydrates released by the three species, particularly in the proportion of monosaccharides. Compositional heterogeneity among carbohydrates appears to be the rule among phytoplanktonic species (Paulsen and Vieira, 1994). Myklestad (Mykletad, 1995) discussed the variability in the composition of carbohydrates released by phytoplankton, proposing it is a specie-specific process. Even species from the same genera display EPS with highly dissimilar compositions. Paulsen and Vieira (Paulsen *et al.*, 1992) characterized the sulfated EPS released by a soil *Cryptomonas* sp., which is different from the EPS released by the *C. tetrapyrenoidosa* shown here and from a tropical strain of *Cryptomonas obovata* (Giroldo and Vieira, 2002).

Although the amounts of carbohydrates released had been higher in *S. orbiculare*, an increase in the release rate

Table IV: Composition of the carbohydrates released by Staurastrum orbiculare during the growth evaluated by high performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC)

Sugars	Fraction	Exponential growth–initial (7 days)		Exponential growth–final (40 days)		Stationary growth (60 days)	
		% 1	% 2	% 1	% 2	% 1	% 2
Fucose	1	98.6	47.2	99.8	65.5	99.9	70.8
	2	1.4	63.8	0.2	2.6	0.1	2.0
	Total	100	47.4	100	62.4	100	69.5
Rhamnose	1	100	2.5	83.0	6.9	90.8	5.6
	2	0	0	17.0	26.3	9.2	30.6
	Total	100	2.5	100	7.9	100	6.1
Arabinose	1	100	2.0	100	4.1	98.9	4.7
	2	0	0	0	0	1.1	2.7
	Total	100	2.0	100	3.9	100	4.7
Galactose	1	100	7.4	94.1	8.5	97.3	9.4
	2	0	0	5.9	9.9	2.7	13.7
	Total	100	7.3	100	8.7	100	9.4
Glucose	1	98.9	34.4	75.6	9.1	83.3	4.0
	2	1.1	36.4	27.4	61.2	16.7	42.4
	Total	100	34.4	100	11.5	100	4.7
Man/Xyl	1	100	6.5	100	5.8	97.2	5.5
	2	0	0	0	0	2.8	8.6
	Total	100	6.4	100	5.6	100	5.6
Total	1	99.0	100	94.9	100	98.2	100
	2	1.0	100	5.1	100	1.8	100
	Total	100	100	100	100	100	100

Fraction 1, high molecular weight (HMW) carbohydrates and fraction 2, free carbohydrates. % 1 shows the distribution of the monosaccharides in each fraction and % 2 shows the percentage of the monosaccharides in the total carbohydrates for each fraction.

during the stationary growth phase was observed in the three species. The same phenomenon was found in several studies on different microalgal species from various environments (Vieira and Myklestad, 1986; Lombardi *et al.*, 1998; Alcoverro *et al.*, 2000; Brouwer and Stal, 2002). This process has remarkable significance on the carbon cycling in natural waters. It could be related to accumulation patterns of DOC in surface waters after spring phytoplanktonic blooms (Carlson *et al.*, 1994; Biddanda and Cotner, 2002). The increase of polysaccharide production on the stationary phase has been ascribed to the absence of cell division and the release of carbohydrates would be supported by photosynthesis. Although photosynthesis would also be affected by nutrient limitation, it should be active enough to produce carbohydrates or other carbon-rich compounds at least initially. The absence of a decrease in POC cell^{-1} during the growth phases confirmed that internal carbon reserve was maintained and the excess of photoassimilated carbon was diverted to DOC and extracellular carbohydrate production. It was also confirmed by the significant increase in the CHO/POC relation in all cultivated species, evidencing the increase of the cell investment in the extracellular carbohydrate production at the stationary growth phase. This scenario was firstly proposed by Fogg (Fogg, 1983): environmental nutrient limitation conditions would decrease the population growth and the active photosynthesis would provide carbon-rich molecules in excess, such as polysaccharides. The release of this carbon is needed to prevent the mechanical effects of the accumulation of excess photosynthate and also minimize the extent of photo-oxidation (Fogg, 1983; Smith and Underwood, 2000).

This explanation, however, does not suffice to elucidate the complexity of carbohydrate release. Excess carbon for the protection of the photoapparatus might be disposed of through the release of less heterogeneous and complex compounds. If the polysaccharides are released specifically to perform these functions, our knowledge of the meaning of such complexity is as yet insufficient. The remarkable heterogeneity, variability and size (often

Table V: Composition of the carbohydrates released by Thalassiosira sp. during the growth evaluated by high performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC)

Sugars	Fraction	Exponential growth–initial (7 days)		Exponential growth–final (14 days)		Stationary growth (25 days)	
		% 1	% 2	% 1	% 2	% 1	% 2
Fucose	1	76.2	11.4	82.5	17.2	87.3	23.6
	2	23.8	13.9	11.6	32.5	7.2	21.5
	3	0	0	5.9	14.3	5.5	31.3
	Total	100	10.6	100	18.0	100	23.7
Rhamnose	1	100	10.0	100	11.0	89.6	11.6
	2	0	0	0	0	10.4	14.9
	3	0	0	0	0	0	0
	Total	100	7.1	100	9.4	100	11.3
Galactose	1	47.1	5.7	85.7	3.6	89.6	4.5
	2	52.9	25.1	14.3	8.1	10.4	5.8
	3	0	0	0	0	0	0
	Total	100	8.6	100	3.7	100	4.4
Glucose	1	49.2	22.9	58.4	13.4	74.0	20.0
	2	16.9	30.5	9.7	29.7	14.0	42.1
	3	33.9	100	31.9	85.7	12.0	68.7
	Total	100	32.8	100	19.8	100	23.9
Man/Xyl	1	86.4	50	96.1	54.8	96.6	40.3
	2	13.6	30.5	3.9	29.7	3.4	15.7
	3	0	0	0	0	0	0
	Total	100	40.9	100	49.1	100	36.7
Total	1	70.7	100	86.2	100	87.9	100
	2	18.2	100	6.5	100	7.9	100
	3	11.1	100	7.3	100	4.2	100
	Total	100	100	100	100	100	100

Fraction 1, high molecular weight (HMW) carbohydrates; fraction 2, intermediary molecular weight (IMW) carbohydrates and fraction 3, free carbohydrates. % 1 shows the distribution of the monosaccharides in each fraction and % 2 shows the percentage of the monosaccharides in the total carbohydrates for each fraction

higher than 2×10^6 D) of these polysaccharides suggest complex roles as well as the protection of photosynthesis. The properties of the polysaccharide released by the three species were probably modified by the alteration of the basic monomeric composition during the experimental period. The hydrophobic components, such as fucose and rhamnose, increased with the advancing age of the cultures. Because so little information is available on such alterations, which may have important implications for the planktonic community, this process should be the focus of future studies.

Our results showed that polysaccharides are the principal extracellular carbohydrate released by phytoplankton, as proposed by Vieira *et al.* (Vieira *et al.*, 1998) and Giroldo and Vieira (Giroldo and Vieira, 1999, 2002). These compounds can be used as a substrate for heterotrophic communities in the surrounding algae. It is now known that bacterioplankton can use complex polysaccharides released by phytoplankton (Freire-Nordi and Vieira, 1996, 1998; Giroldo *et al.*, 2003). A new concept on the bioreactivity of HMW organic compounds in natural waters was proposed by Amon and Benner (Amon and Benner, 1996). The authors demonstrated that HMW compounds are the major substrates for bacterial metabolism, according to their size-reactivity continuum model, in which the larger sized organic matter is more reactive than smaller sized organic matter. However, the data revealed by microbial degradation experiments of phytoplanktonic polysaccharides are insufficient to identify an actual pattern of their degradation kinetics and their utilization as a carbon source by microbial communities. The heterogeneity, as well as the specific composition and structure, suggests significant variation in degradation time and microbial growth efficiency. The bacterial growth efficiency of the EPS from *Staurastrum inversenii* and its maximum degradation rate were 35% and 2.47 day^{-1} (Pacobahyba, 2002), while the

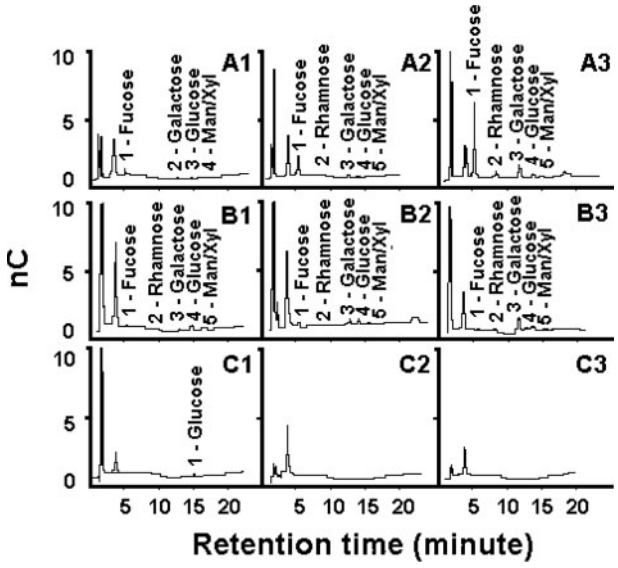

Fig. 3. High performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC) analyze of the carbohydrates released by *Cryptomonas tetrapyrenoidosa* during the growth: (**1**) initial exponential phase, (**2**) final exponential phase and (**3**) stationary phase. (**A**) High molecular weight, (**B**) intermediary molecular weight and (**C**) low molecular weight carbohydrates.

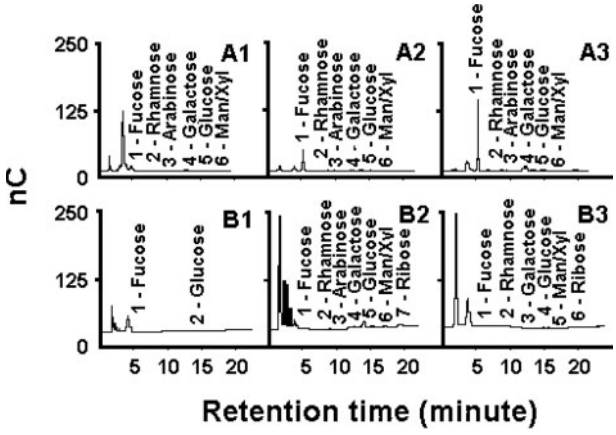

Fig. 4. High performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC) analyze of the carbohydrates released by *Staurastrum orbiculare* during the growth: (**1**) initial exponential phase, (**2**) final exponential phase and (**3**) stationary phase. (**A**) High molecular weight and (**B**) low molecular weight carbohydrates.

maximum degradation rate of the EPS released by *Ankistrodesmus densus* was 1.1 day^{-1}, depending on the fraction (Freire-Nordi and Vieira, 1998). Our calculations showed that the release of carbohydrates by phytoplankton could support bacterial populations, as postulated by Wood and Van Valen (Wood and Van Valen, 1990). The amounts of carbohydrates released by one cell of the three species in the polymeric form could

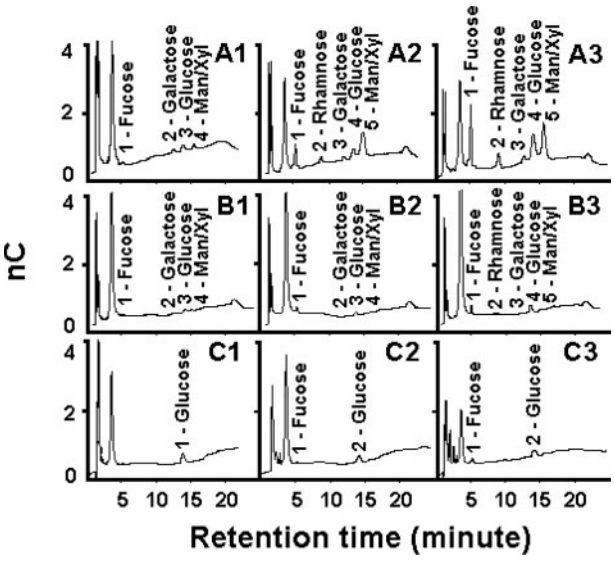

Fig. 5. High performance liquid chromatography equipped with pulse amperometric detection (PAD–HPLC) analyze of the carbohydrates released by *Thalassiosira* sp. during the growth: (**1**) initial exponential phase, (**2**) final exponential phase and (**3**) stationary phase. (**A**) High molecular weight, (**B**) intermediary molecular weight and (**C**) low molecular weight carbohydrates.

support bacterial biomass from 0.01 to 0.38 mg C L^{-1}, while the free sugars can support bacterial biomass up to 0.0014 mg C L^{-1}, according to the estimates of Hanish (Hanish *et al.*, 1996). The species tested in this study were isolated from a tropical eutrophic habitat with dissolved organic carbon concentrations between 8 and 15 mg L^{-1}, and the high bacterial density probably controls the level of available dissolved organic matter pool (Rheinheimer, 1974).

Despite the significance of the carbohydrates released by the phytoplankton, their roles in the environment are still obscure, and it is not clear whether the benefits of this process exceed the loss of carbon for phytoplanktonic cells. A reciprocal association with heterotrophic bacteria would be an interesting mechanism. Polysaccharide released initially to produce capsules or sheaths for algae can be used for bacterial populations, either as a mechanical support or as a carbon source. However, little information exists on the level of specificity of such association (Paerl, 1978; Grossart *et al.*, 2005). Even the soluble or colloidal polysaccharide released into the environment may support an area around the phytoplanktonic cell known as phycosphere (Bell and Mitchell, 1972), whose chemical, physical and biological conditions differ substantially from the surrounding environment. The primary cause of EPS release by phytoplankton may be quite different from its original one and the role of this process has probably become extremely diversified through the evolution of each habitat or specific situation.

ACKNOWLEDGEMENTS

The authors thank FAPESP (Processes 99/01353–5 and 99/07766–0) and CNPq/PRONEX for the financial support of this work. We are also indebted to Dr. Mariangela Menezes for the identification of *C. tetrapyrenoidosa* and MSc Pedro Ortolano and Thais Bittar for the carbon analysis.

REFERENCES

Alcoverro, T., Conte, E. and Mazzella, L. (2000) Production of mucilage by the adriatic epipelic diatom *Cylindrotheca closterium* (Bacillariophyceae) under nutrient limitation. *J. Phycol.*, **36**, 1087–1095.

Amon, M. and Benner, B. (1996) Bacterial utilization of different classes of dissolved organic matter. *Limnol. Oceanogr.*, **41**, 41–51.

Banse, K. (1976) Rates of growth, respiration and photosynthesis of unicellular algae as related to cell size – a review. *J. Phycol.*, **12**, 135–140.

Bell, W. H. and Mitchell, R. (1972) Chemotatic and growth responses of marine bacteria to algal extracellular products. *Biol. Bull.*, **143**, 265–277.

Bell, W. H. and Sakshaug, E. (1980) Bacterial utilization of algae products. 2. A kinetic study of natural population. *Limnol. Oceanogr.*, **19**, 833–839.

Biddanda, B. and Benner, R. (1997) Carbon, nitrogen, and carbohydrate fluxes during the production of particulate and dissolved organic matter by marine phytoplankton. *Limnol. Oceanogr.*, **42**, 506–518.

Biddanda, B. and Cotner, J. (2002) Love handles in aquatic ecosystems: the role of dissolved organic carbon drawdown, resuspended sediments, and terrigenous inputs in the carbon balance of Lake Michigan. *Ecosystems*, **5**, 431–445.

Bjørnsen, P. K. (1988) Phytoplankton exudation of organic matter: why do healthy cells do it. *Limnol. Oceanogr.*, **33**, 151–154.

Brouwer, J. F. C. and Stal, L. J. (2002) Daily fluctuations of exopolymers in cultures of the benthic diatoms *Cylindrotheca closterium* and Nitzschia sp. (Bacillariophyceae). *J. Phycol.*, **38**, 464–472.

Calijuri, M. C. (1999) A comunidade fitoplanctônica em um reservatório tropical (Barra Bonita, SP). Full Professor Thesis. University of Sao Paulo, Brazil, 211 pp.

Calijuri, M. C., Dos Santos, A. C. A. and Jati, S. (2000) Temporal changes in the phytoplankton community structure in a tropical and eutrophic reservoir (Barra Bonita, SP – Brazil). *J. Plankton Res.*, **24**, 617–634.

Carlson, C., Ducklow, H. and Michaels, A. (1994) Annual flux of dissolved organic carbon from the euphotic zone in the northwestern Sargasso Sea. *Nature*, **371**, 405–408.

Domozych, D. S. and Domozych, C. R. (1993) Mucilage processing and secretion in the green alga *Closterium*. II. Ultrastructure and immunucytochemistry. *J. Phycol.*, **29**, 659–667.

Dubois, M., Guilles, K. A., Hamilton, J. K. *et al.* (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356.

Fogg, G. E. (1975) *Algae Cultures and Phytoplankton Ecology*. The University of Wisconsin Press, Wisconsin.

Fogg, G. E. (1983) The ecological significance of extracellular products of phytoplankton photosynthesis. *Bot. Mar.*, **26**, 3–14.

Freire-Nordi, C. S. and Vieira, A. A. H. (1996) Utilization of extracellular polysaccharides from *Ankistrodesmus densus* Kors (Chlorophyceae) by heterotrophic bacteria. *Rev. Microbiol.*, **27**, 27–32.

Freire-Nordi, C. S. and Vieira, A. A. H. (1998) Degradability of polysaccharides compounds excreted by *Ankistrodesmus densus* Kors (Chlorophyceae) in cultures of natural bacterial communities. *Verh. Int. Verein. Limnol.*, **26**, 1685–1688.

Giroldo, D. and Vieira, A. A. H. (1999) Assimilation and release of 14C in a tropical strain of *C. obovata* (Cryptophyceae) exposed to several irradiances. *J. Plankton Res.*, **21**, 1911–1921.

Giroldo, D. and Vieira, A. A. H. (2002) An extracellular sulphated fucose-rich polysaccharides produced by a tropical strain of *C. obovata* (Cryptophyceae). *J. Appl. Phycol.*, **14**, 185–191.

Giroldo, D., Vieira, A. A. H. and Paulsen, B. S. (2003) The relative increase of deoxy sugars during microbial degradation of an extracellular polysaccharide released by a tropical freshwater *Thalassiosira* sp (Bacillariophyceae). *J. Phycol.*, **39**, 1109–1115.

Gremm, T. J. and Kaplan, L. A. (1997) Dissolved carbohydrates in streamwater determined by HPLC-PAD and pulse amperometric detection. *Limnol. Oceanogr.*, **42**, 385–393.

Grossart, H. P., Levold, F., Allgaier, M. *et al.* (2005) Marine diatom species harbor distinct bacterial communities. *Environ. Microbiol.*, **7**, 860–873.

Guillard, R. R. L. and Lorenzen, C. J. (1972) Yellow-green algae with chlorophyllide c. *J. Phycol.*, **8**, 10–14.

Hanish, K., Schweitzer, M. and Simon, M. (1996) Use of dissolved carbohydrates by planktonic bacteria in a mesotrophic lake. *Microbial Ecol.*, **31**, 41–55.

Klaveness, D. (1985) Ecology of Cryptomonadida: a first review. In Sondgren, C. D. (ed.), *Growth and Reproductive Strategies of Freshwater Phytoplankton*. Cambridge University Press, Cambridge, pp. 105–133.

Lancelot, C. (1984) Extracellular release of small and large molecules by phytoplankton in the southern Bight of the North Sea. *Estuar. Coas. Shelf. Sci.*, **18**, 65–77.

Lombardi, A. T., Vieira, A. A. H. and Sartori, A. L. (1998) Extracellular carbohydrate production by *Micrasterias furcata* (Desmidiaceae) grown in various nitrate concentration. *Hoehnea*, **25**, 1–9.

Mayzaud, P., Chanut, J. P. and Ackman, R. R. (1989) Seasonal changes of the biochemical composition marine particulate matter with special reference tp fatty acids and sterols. *Mar. Ecol. Progr. Ser.*, **56**, 189–204.

Myklestad, S. M. (1995) Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *Sci. Total Environ.*, **165**, 155–164.

Pacobahyba, L. D. (2002) Decomposição de *Staurastrum*c.f. *iversenii* Nygaard var. *americanum*: efeitos da qualidade do recurso, da disponibilidade de oxigênio e da temperatura. PhD Thesis. Federal University of São Carlos, Brazil, 158 pp.

Paerl, H. W. (1978) Role of bacteria in promoting N_2 fixation by *Anabaena* sp in aquatic habitats. *Micobial Ecol.*, **4**, 215–231.

Paulsen, B. S. and Vieira, A. A. H. (1994) Structural studies on extracellular dissolved and capsular polysaccharides produced by *Spondylosium panduriforme*. *J. Phycol.*, **30**, 638–641.

Paulsen, B. S., Vieira, A. A. H. and Klaveness, D. (1992) Structure of extracellular polysaccharides produced by a soil *Cryptomonas* sp. (Cryptophyceae). *J. Phycol.*, **28**, 61–63.

Rheinheimer, G. (1974) *Aquatic Microbiology*. Jonh Wiley and Sons, London, 184 pp.

Sheldon, R. W. (1984) Phytoplankton growth rates in tropical ocean. *Limnol. Oceanogr*., **29**, 1342–1346.

Smith, D. J. and Underwood, G. J. C. (2000) The production of extracellular carbohydrates by estuarine benthic diatoms: the effects of growth phase and light and dark treatment. *J. Phycol*., **2**, 321–333.

Soares, A. (2003) Qualidade da água e fluxos de nutrientes na interface sedimento-água nas represas do rio Tietê. MSc Dissertation. Federal University of São Carlos, Brazil, 86 pp.

Vieira, A. A. H. (1983) Purification of phytoplankton cultures with Dakin solution. *Rev. Microbiol*., **14**, 202–203.

Vieira, A. A. H., Lombardi, A. T. and Sartori, A. L. (1998) Release of dissolved organic matter in a tropical strain of *Synura petersenii* (Chrysophyceae) grown under high irradiances. *Phycologia*, **37**, 357–362.

Vieira, A. A. H. and Myklestad, S. (1986) Production of extracellular carbohydrate in cultures of *Ankistrodesms densus*. *J. Plankton Res*., **8**, 985–994.

Wood, A. M. and Van Valen, L. M. (1990) Paradox lost? On the release of energy-rich compounds by phytoplankton. *Mar. Microbial Foods Webs*, **4**, 103–116.