J. Phycol. **39,** 1109–1115 (2003)

RELATIVE INCREASE OF DEOXY SUGARS DURING MICROBIAL DEGRADATION OF AN EXTRACELLULAR POLYSACCHARIDE RELEASED BY A TROPICAL FRESHWATER *THALASSIOSIRA* SP. (BACILLARIOPHYCEAE)[1]

Danilo Giroldo,[2] *Armando Augusto Henriques Vieira*

Universidade Federal de São Carlos, Departamento de Botanica, Laboratorio de Ficologia, Via Washington Luis Km 235, São Carlos, SP, Brazil, 13565-905

and

Berit Smestad Paulsen

University of Oslo, School of Pharmacy, Department of Pharmacognosy, P.O. Box 1068 Blindern, 0316 Oslo, Norway

The aim of this study was to characterize the extracellular polysaccharides (EPS) released by a freshwater *Thalassiosira* sp. (Bacillariophyceae) and evaluate their degradation by heterotrophic microbial populations from the same habitat of *Thalassiosira* sp., a tropical eutrophic reservoir. The EPS were purified by anion exchange column chromatography, the monosaccharide composition was determined by GC, and the linkages of the monosaccharides by GC-MS. The EPS is a mannose-rich heteropolysaccharide composed of two different acidic fractions. Both of these fractions are composed of mannose, rhamnose, fucose, xylose, galactose, glucose, glucuronic acid, and *N*-acetyl glucosamine but with different proportions. *N*-acetyl galactosamine occurs only in fraction 1 and galacturonic acid only in fraction 2. We monitored the concentrations of the monosaccharides in the EPS during its degradation using pulse amperometric detection in an HPLC. The decay patterns of the monosaccharides were varied and the deoxy sugars, fucose and rhamnose, were degraded at a slower rate than the other components, increasing their relative concentrations and the hydrophobic feature of the EPS. The possibility of a selective degradation, which enhances the stickiness of the EPS, promoting transparent exopolymeric particles and aggregate formation, is discussed based on the literature data.

Key index words: **carbohydrate release; diatom; EPS; microbial degradation, *Thalassiosira***

Abbreviations: **EPS, extracellular polysaccharide; PAD, pulse amperometric detection; TEP, transparent exopolymeric particles**

The release of energy-rich compounds by phytoplanktonic organisms is well known both in seawater and freshwater (Fogg 1983). Carbohydrates, mainly extracellular polysaccharides (EPS), compose most of this extracellular material both in cultures (Myklestad 1995) and in natural waters (Jørgensen and Jensen 1994, Gremm and Kaplan 1997). Although polysaccharides are considered more refractory to degradation by heterotrophic bacteria than low molecular weight carbohydrates, the utilization of these compounds by bacteria has been demonstrated in the literature (Chrost and Faust 1983, Kato and Stabel 1984). Moreover, bacterial populations may be supported by phytoplanktonic EPS when provided as the only carbon source in laboratory experiments (Freire-Nordi and Vieira 1996, 1998). The relevance of the polysaccharides in the microbial loop, as well as in the formation of aggregates and transparent exopolymeric particles (TEP) (Passow et al. 1994), places these compounds in a key position for understanding ecosystem processes in the aquatic environment.

EPS release by phytoplankton is stimulated by environmental conditions such as nutrient deficiency (Vieira and Myklestad 1986, Giroldo and Vieira 2002), producing an apparent paradox: Nutrient-limited phytoplankton increases the carbon supply to heterotrophic bacteria, their potential competitor (Bratbak and Thingstad 1985). Nutrient regeneration from bacterial growth and disposal of photoassimilated carbon in excess (Fogg 1983, Wood and Van Valen 1990) are some suggestions of possible advantages for phytoplanktonic cells resulting from carbohydrate release. Nevertheless, protection of the photosynthetic apparatus by an overflow mechanism has become more evident with the advancement of fluorescent techniques for measurements of photophysiological responses in algae (Smith and Underwood 2000, Perkins et al. 2001).

The data available on the composition of phytoplanktonic EPS as well as EPS utilization by bacteria are quite rare, considering the number of taxa, and are especially sparse for planktonic freshwater diatoms. Most results concern biofouling organisms (Cooksey and Cooksey 1985, Wustman et al. 1997) or epipelic diatoms (Smith and Underwood 1998, Underwood and Smith 1998, Staats et al. 1999). The structure and composition of the EPS have remarkable influence on

[1] Received 10 January 2003. Accepted 13 July 2003.

[2] Author for correspondence: e-mail pdanilo@iris.ufscar.br.

their physical and chemical properties, defining many of the features of such EPS. The kinetics of degradation, the TEP, and aggregate formation capacity and the level of bacterial association could be related to the EPS characteristics, highlighting the significance of studies focused on the composition and structure of phytoplanktonic EPS.

There is little information in the literature regarding alterations in the composition of EPS released by phytoplanktonic algae. Besides genetic control, variations can occur during biosynthesis as a deviation from specificity of the transferases or errors in the polymerization (Leppard 1995). However, the environmental factors, such as a selective microbial degradation (Aluwihare and Repeta 1999), can also alter EPS composition and structure. Consequently, chemical and physical properties of extracellular compounds, such as hydrophilic and hydrophobic features, can also be controlled by the microbial activity. Here we characterize the composition of an EPS released by a tropical freshwater planktonic diatom, *Thalassiosira* sp., and determine the linkages of the monosaccharides. We also calculate the coefficient of degradation of EPS monosaccharides by microbial activity to observe the alteration of the EPS composition by selective degradation.

MATERIALS AND METHODS

Organisms and culture conditions. Thalassiosira sp. and the ambient heterotrophic microbial community were collected in the Barra Bonita reservoir, located in the Tiete River, SP, Brazil (22°29′S, 48°34′W). This is a eutrophic human-made lake built mainly to generate electricity. The surface area is 324.8 km^2 and the maximum depth is 30 m, corresponding to a maximum volume of 3.1×10^6 m^3 (Tundisi and Matsumura-Tundisi 1990). *Thalassiosira* sp. was isolated directly by microscopy, and axenic cultures were obtained by washing with Dakin™ solution (Vieira 1983). The culture medium was complete WC (Guillard and Lorenzen 1972) modified by the addition of 150 μM Na_2SiO_3. 9 H_2O (WC silica plus), and the cultures were kept under 100 μmol photons · $m^{-2} \cdot s^{-1}$ (PAR) at 22 ± 1° C and in a dark:light cycle of 12:12 h. Tests to check for bacterial contamination in the axenic cultures were performed regularly with WC silica plus medium modified by the addition of glucose and peptone (250 mg · L^{-1} per test).

Thalassiosira *sp. growth.* A 9-L carboy filled with 7 L of WC silica plus medium was inoculated with a *Thalassiosira* sp. inoculum (300 mL, exponential growth phase, approximately 10^6 cells · mL^{-1}) and cultivated in duplicate under the above-described conditions. The cultures were bubbled with filtered air humidified in sterilized and acidified distilled water (pH 2.0). 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. A 5-mL fraction was fixed with Lugol to determine the cell density by direct counts (duplicate) on microscope (Zeiss Axioplan 2, Jena, Germany). The duplication time was calculated using the equation described by Fogg (1975).

EPS isolation and characterization. After approximately 30 days of growth, *Thalassiosira* sp. axenic cultures (7 L) were filtered by tangential flow through a hollow fiber cartridge with 0.65 μm pore (A/G Technology, Needham, MA, USA). The filtrate was concentrated in a rotary evaporator at 40° C and dialyzed against distilled water in dialysis tubes with a 12- to 14-kDa molecular weight cut-off. The dialyzed material was freeze dried and stored at –4° C under nitrogen. The freeze-dried material was further purified by anion exchange column chromatography and analyzed by GC and electron impact MS after derivatizations as described below. The anion exchange column chromatography was performed using the batch separation method, without gradient, under the following conditions: gel = Sepharose DEAE fast flow (Pharmacia, Peapack, NJ, USA) in chloride form, bed dimensions = 2.5×15 cm, flow rate = 1 mL · 3 min^{-1}, eluent I = distilled water, eluent II = 0.5 M NaCl, eluent III = 1.0 M NaCl, and eluent IV = 2.0 M NaCl, running at the ambient temperature. Sodium azide (0.007 M) was used to avoid bacterial contamination. The column was first regenerated with 2.0 M NaCl and washed with distilled water. The sample was applied and eluted with distilled water to obtain the neutral fraction. Elution with 0.5 M NaCl then yielded the weak acid fraction, and finally elution with 1.0 M NaCl was used to obtain the strong acid fraction. Carbohydrates were detected in the 3-mL fractions by the phenol-sulfuric method (Dubois et al. 1956). The fractions with significant amount of carbohydrates were pooled, dialyzed against distilled water, and freeze dried. The monosaccharide composition of the EPS was determined by GC of the trimethylsilylated derivatives of the methyl-glycosides obtained by methanolysis using 4 M HCl in methanol at 80° C for 24 h (Reinhold 1972, Barsett et al. 1992). Mannitol was used as an internal standard. Methylation of the polymers was carried out to determine the linkages of the monosaccharides (Kim and Carpita 1992), followed by gas chromatographic analysis coupled with electron impact MS of the derived partially methylated alditol acetates (Barsett et al. 1992, Samuelsen et al. 1995). The fractions with more than 5% of uronic acids were reduced to the corresponding neutral sugars before the methylation (Sims and Bacic 1995). Protein content was determined in the fractions by the Lowry method (Lowry et al. 1951).

Microbial degradation of the EPS. The microbial inoculum was obtained from the euphotic zone (0–5 m, determined by a Spherical Quantum Sensor LI-193SA and an LI-250 Light Meter, LiCor, Lincoln, NE, USA). The sample was aseptically filtered at low vacuum pressure (10 mm Hg · cm^{-1}) through calcinated glass fiber prefilters AP-20 (Millipore, Dublin, CA, USA) to remove particles, zooplankton, and phytoplankton. Fifty milliliters of this sample was inoculated in 1 L culture medium prepared as follows: 0.5 L WC medium + 0.5 L water from the Barra Bonita reservoir + EPS (100 mg · L^{-1}, final concentration). The water from Barra Bonita reservoir included in the culture medium was filtered through 0.45-μm prewashed acetate membranes (Millipore). A control composed of culture medium without EPS was inoculated with the microbial community and served as a reference.

The experiments (two replicates plus control) were carried out in the dark at 25° C for 35 days. Samples were taken at 7-day intervals for analysis of EPS degradation. They were filtered at low vacuum pressure (10 mm Hg · cm^{-1}) through 0.45-μm acetate membranes. The EPS degradation was first evaluated by gel filtration column chromatography, using Sepharose CL-6B gel (Pharmacia; exclusion limits 10^6–10^4 D). Gel filtration column chromatography was used to monitor the transformation of the EPS into lower molecular weight carbohydrates by microbial activity. The gel filtration chromatography was performed under the following conditions: bed dimensions = 1.5×50 cm, flow rate = 1 mL · 7 min^{-1}, eluent = distilled water + 2% butanol, sample volume = 5% of total volume (6 mL), running at ambient temperature. Standard dextrans 10^4 and 2×10^6 D were used as references. Carbohydrates were detected in the 5-mL fractions by the phenol-sulfuric method. The fractions with a significant amount of carbohydrates were pooled, hydrolyzed, and analyzed by pulse

amperometric detection (PAD) in HPLC (Gremm and Kaplan 1997). The PAD-HPLC analysis were performed on a Dionex (Sunnyvalle, CA, USA) DX500, consisting of a PEEK version GP40 gradient pump module, an ED40 electrochemical detector, and an LC5 manual injector with a Rheodyne 9125 valve and a 25-μL peek sample loop. The ED40 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 an appropriate guard column (4×50 mm), was used to separate the monosaccharides. The eluent for the separation was 18 mM NaOH, and regeneration of the column used 200 mM NaOH at a flow rate of $1\,\text{mL} \cdot \text{min}^{-1}$. All samples were desalted on BioRad (Hercules, CA, USA) ionic exchange resin (AG2X8, anion exchange, and AG50W, cation exchange) to remove salts from culture media.

A first-order decay equation was used to describe the EPS degradation following the reaction presented below:

$$\text{EPS} \rightarrow CO_2 + \text{particulate organic carbon (microbial biomass)}$$

The equation assumes the reaction rates are proportional to the reagent amounts and define a constant whose unit is day^{-1}. The decay coefficients of the total polysaccharide and each monomer were calculated using the following equation:

$$A_t = A_0 e^{-kt}$$

where A_t is the carbohydrate concentration, A_0 is the initial carbohydrate concentration, k is the decay coefficient (day^{-1}), e is the base of natural logarithms, and t is time.

The temporal variations of the EPS concentration were used to determine the decay coefficients. For this purpose a nonlinear regression method was used (Levenberg-Marquardt algorithm), according to Press et al. (1993). The aim of this study was not to describe in detail the EPS degradation process using more complex equations and reactions. The parameterization showed above was used only to compare the degradation pattern of each EPS component, and the first-order equation was chosen because of its practicality.

Bacterial growth was evaluated by direct counts on epifluorescence light microscope with UV and light source (Zeiss Axioplan 2), after staining the cells using 4′6′-diamino-2-phenylindole (Porter and Feig 1980).

RESULTS

Thalassiosira sp. growth. Figure 1 shows the growth of *Thalassiosira* sp. during 25 days. The doubling time was 1.7 days until day 10, when it decreased greatly to 24 days, characterizing a transition between exponential and stationary phases. At the end of the experiment, we obtained approximately 100 mg of polysaccharide material from approximately $1.5 \times 10^5\,\text{cells} \cdot \text{mL}^{-1}$.

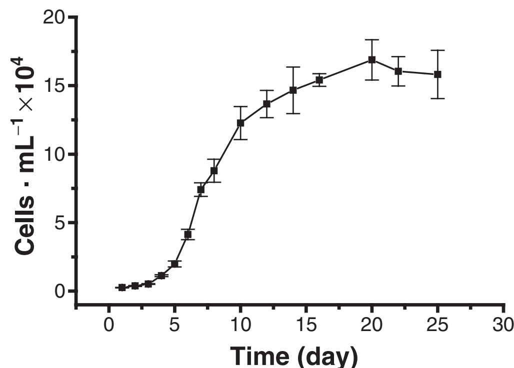

FIG. 1. *Thalassiosira* sp. axenic growth in WC/C silica plus during 25 days under an irradiance of 100 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and 22 ± 1° C. The bars show standard variation ($n = 4$).

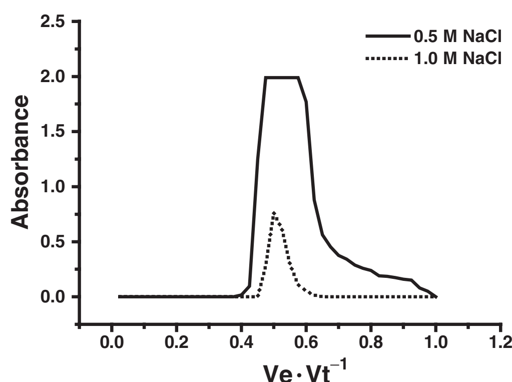

FIG. 2. Anion exchange column chromatography (Pharmacia DEAE Sepharose fast flow) of the total polysaccharide released by *Thalassiosira* sp. Fraction 1: Eluted with 0.5 M NaCl. Fraction 2: Eluted with 1.0 M NaCl.

EPS characterization. Figure 2 shows the anion exchange column chromatography of the EPS released by *Thalassiosira* sp. The major fraction was eluted with 0.5 M NaCl (fraction 1), and a small fraction was eluted with 1 M NaCl (fraction 2). Although the two fractions were composed mainly of mannose, the actual composition was different (Table 1), especially in the proportion of rhamnose, xylose, uronic acids, and amino sugars. A very small amount (less than 0.01 mg) of a neutral fraction was detected in the polysaccharide. These were probably glucose-rich compounds related to intracellular material from cell death and did not comprise the main EPS. No EPS material was obtained during elution with 2.0 M NaCl.

Table 2 shows the glycosidic linkages of the components of the two fractions. Fraction 1 was essentially

TABLE 1. Carbohydrate composition (%) determined by GC analysis of the polysaccharide released by *Thalassiosira* sp., after methanolysis and TMS derivatization of the fractions obtained by anion exchange column chromatography.

	Fraction 1	Fraction 2
Rhamnose	18.3%	3.3%
Fucose	7.7%	2.1%
Xylose	6.2%	19.5%
Mannose	51.8%	57.4%
Galactose	4.4%	4.2%
Glucose	3.8%	0.8%
Glucoronic acid	1.7%	5.5%
Galacturonic acid	0%	4.9%
N-acetyl-galactosamine	2.0%	0%
N-acetyl-glucosamine	3.8%	2.0%

Fraction 1, eluted with 0.5 M NaCl; fraction 2, eluted with 1.0 M NaCl.

TABLE 2. Glycosidic linkages of the monosaccharides present in the original polysaccharides determined by GC-MS of the corresponding partly methylated alditol acetates.

	Linkages	F1 %M	F1 %T	F2 %M	F2 %T
Rhamnose	T	10.7	1.9	0	0
	1,2	12.6	2.4	0	0
	1,3	69.1	12.6	100	3.3
	1,2,4	4.4	0.8	0	0
	1,3,4	3.2	0.6	0	0
	Total	100	18.3	100	3.3
Fucose	T	27.3	2.1	0	0
	1,3	72.7	5.6	100	2.1
	Total	100	7.7	100	2.1
Xylose	T	48.0	3.0	32.0	6.2
	1,2	37.7	2.3	0	0
	1,4	0	0	68.0	13.3
	1,2,4	14.3	0.9	0	0
	Total	100	6.2	100	19.5
Mannose	T	30.6	15.9	20.5	11.7
	1,2	11.6	6.0	18.0	10.3
	1,3	6.1	3.2	0	0
	1,4	14.9	7.7	61.5	35.4
	1,3,4	6.6	3.5	0	0
	1,4,6	20.8	10.8	0	0
	1,3,4,6	9.4	4.9	0	0
	Total	100	52.0	100	57.4
Galactose	T	73.1	3.3	33	1.4
	1,2	24.9	1.1	0	0
	1,4	0	0	67	2.8
	Total	100	4.4	100	4.2
Glucose	T	56.5	2.1	0	0
	1,2	43.5	1.7	0	0
	Total	100	3.8	0	0
Glucoronic ac.	T	0	0	100	5.5
	Total	0	0	100	5.5
Galacturonic ac.	T	0	0	100	4.5
	Total	0	0	100	4.5

The percentages of each linkage in the same monosaccharide (%M) and the percentage of the linkage in the total polysaccharide (%T) are shown for the two fractions of the polysaccharide. F1, fraction eluted in the anion exchange chromatography with 0.5 M NaCl; F2, fraction eluted in the anion exchange chromatography with 1.0 M NaCl.

composed by terminal mannose, 1,4,6-linked mannose, and 1,3-linked rhamnose. The mannose linkages were quite complex in this fraction with seven different positions. This polysaccharide has a central branched portion, composed mainly by 1,4,6-linked mannose, and linear chains composed by rhamnose, fucose, mannose, galactose, xylose, and glucose, besides significant portions of terminal mannose. Fraction 1 contained only 2% protein. Fraction 2 showed a less complex pattern than fraction 1. Protein accounted for 6% of fraction 2, and the polysaccharide part of this fraction was linear because of the absence of branch points. The uronic acids were placed in a terminal position, probably supported by a base composed of protein and amino sugars. The principal linkages were 1,4-linked mannose and 1,4-linked xylose. Because fraction 2 was obtained in very low amounts (less than 1 mg), a loss of some components of this fraction during the methylation process may be considered. On the other hand, it is also plausible that this fraction only occurs in the form of small linear chains.

EPS degradation. Figure 3 shows EPS degradation measured by gel filtration column chromatography. The EPS was mainly eluted in one peak between 0 and $0.25\,Ve \cdot Vt^{-1}$ (eluted volume: total column volume), near the reference Blue Dextran (2×10^6 D). Small amounts, probably fragments of the main polysaccharide, were eluted around $0.5\,Ve \cdot Vt^{-1}$. During the experimental period (35 days), no transformation of the EPS in lower molecular weight compounds was observed, but the amount of high molecular weight material was reduced considerably.

Mannose and xylose were not well separated by the PA-10 column because the PAD-HPLC was performed without a postcolumn system. The uronic acids and amino sugars, less than 1% of the EPS, were not

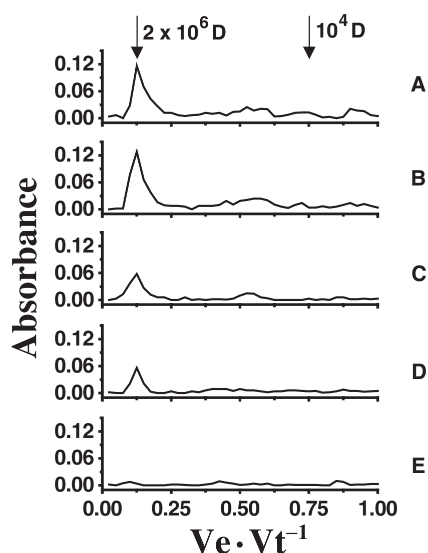

FIG. 3. Bacterial degradation of the total EPS released by *Thalassiosira* sp. measured by gel filtration column chromatography (Pharmacia CL6B). The arrows indicate the standard dextrans used to calibrate the column: 2×10^6 and 10^4 D. A, B, C, D, and E show, respectively, 0, 7, 14, 21, and 35 days after the microbial community inoculation.

measured in the PAD-HPLC because the desalting columns retained them. Though only two replicates were performed in this experiment, the coefficient of variation of the carbohydrate concentration averages were below 10%. Figure 4 shows the decay kinetics of the EPS, besides the decay coefficient of each component of the polysaccharide. The decay coefficients of fucose, rhamnose, and galactose were remarkably slower than those of man/xyl and glucose. Figure 5 shows the increase of the relative concentration of fucose and rhamnose by a selective degradation of the EPS during the experimental period. The sum of the percentages of fucose, rhamnose, and galactose in the EPS increased from 38% to 69.6% of the total

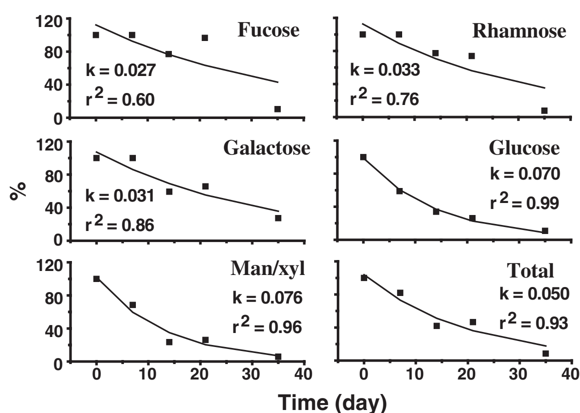

FIG. 4. Bacterial degradation of the EPS released by *Thalassiosira* sp. measured by PAD-HPLC. The decay coefficients are shown for each monosaccharide, which compose the EPS, and also for the total EPS.

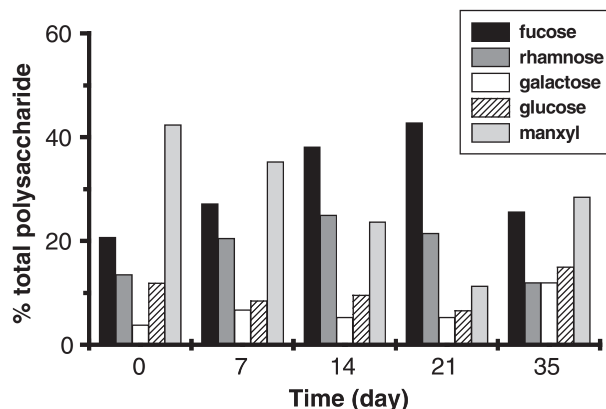

FIG. 5. Qualitative alterations in the *Thalassiosira* sp. EPS composition during the microbial degradation period. The results were obtained using PAD-HPLC analysis, and the sum of the monosaccharides is 100% for each day.

sugars detected in the PAD-HPLC after 21 days. During the last period, from 21 to 35 days, the deoxy sugars and galactose were degraded more effectively than at the beginning of the experiment, decreasing the sum of their percentages to 49.6% of the total polysaccharide.

Bacterial growth. Bacterial growth was significant (Fig. 6), increasing from 10^5 to 10^8 cells · mL^{-1}, reaching the maximum density at 21 days of incubation (8×10^8 bacteria · mL^{-1}). The growth was related to the degradation of the whole EPS, which decreases approximately from 22 to 2 mg · L^{-1}. The carbohydrate concentration in the controls was constant during the incubation period and did not reach more than 1 mg · L^{-1}. The carbohydrate concentration

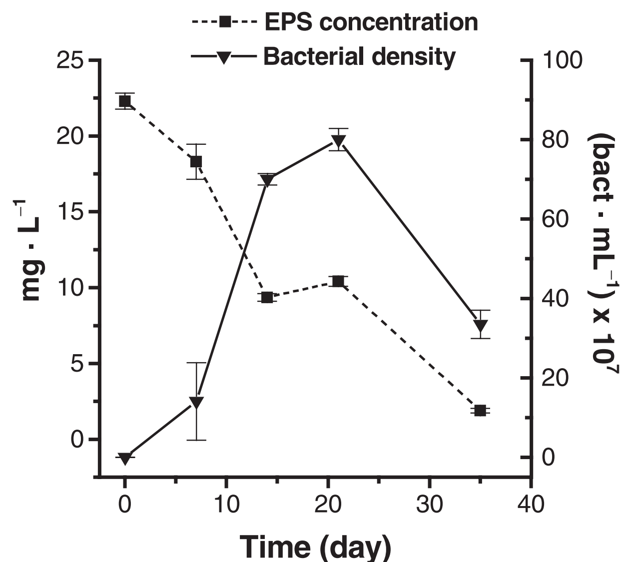

FIG. 6. Bacterial growth (bacteria · mL^{-1}) and *Thalassiosira* sp. EPS concentration (mg · L^{-1}) during microbial degradation period (35 days). The bars show standard variation ($n = 2$).

found in the experiment was smaller than the $100\,mg \cdot L^{-1}$ added in the culture medium because of the hydrolysis recovery (approximately 50%), as well as the exclusion of uronic acids, amino sugars, and proteins (approximately 15%). The rest (approximately 13%) was eluted in the gel filtration column chromatography as fragments of lower molecular weight, and they were discarded in this quantification.

DISCUSSION

Thalassiosira sp. produces a very complex EPS when compared with literature data on EPS from other species (Taylor et al. 1999, Li et al. 2001). We identified 21 different types of linkages in the weak acidic fraction (fraction 1) and 11 in the strong acidic fraction (fraction 2). Despite this complexity, the heterotrophic microbial community from Barra Bonita reservoir was able to degrade the entire polysaccharide complex in about 1 month. Freire-Nordi and Vieira (1996, 1998) found the same behavior in degradation experiments of polysaccharides released by *Ankistrodesmus densus* (Chlorococcales). However, these authors did not investigate the influence of the degradation on the composition of the polysaccharide, and no studies correlating the structure to the degradation of phytoplanktonic polysaccharides are known.

Our results show that the components of the polysaccharide were not degraded in the same pattern. Although most of the glucose and mannose were placed in a terminal position, their high decay rates cannot be related exclusively to the structure of the polysaccharide. About 70% of the galactose was in a terminal position and the degradation rate of galactose was as that of fucose and rhamnose, which occurred mainly in 1,3-linked chains. The results are consistent with the microbial community preferentially attacking specific regions of the polysaccharide initially, and especially those areas rich in glucose and mannose/xylose units. Aluwihare and Repeta (1999) characterized the EPS produced by a marine strain of *Thalassiosira weissflogii*, which is as complex as the one presented here, but the freshwater *Thalassiosira* sp. EPS contained a major proportion of deoxy sugars. These authors also identify a selective microbial degradation pattern of such EPS, in which mannose was degraded faster than rhamnose, corroborating our results. However, the authors did not correlate the relative increase of the deoxy hexoses during the degradation period to the coagulation theory, although they found indications of the flocculation processes in their results.

Many authors correlated the release of extracellular carbohydrates by marine diatoms to the formation of TEPs and aggregates (Kiørboe and Hansen 1993, Passow et al. 1994). This particulate material provides a surface for microbial attachment and would increase the bacterial growth around the diatoms (Middelboe et al. 1995). Such association may promote diatom growth by increasing nutrient regeneration locally (Azam and Smith 1991). The degradation pattern observed in our results has remarkably changed the monosaccharide proportion in the EPS released by *Thalassiosira* sp. during microbial activity. The relative increase of the deoxy sugars, fucose and rhamnose, may also increase the hydrophobic feature of the EPS because of the carbon 6 methyl group. Such hydrophobic properties may promote the TEP and aggregate formation and, considering that aggregation is a part of the life strategy of many diatom species (Gotschalk and Alldredge 1989), the structure of this polysaccharide may promote aggregation by cell collision (Kiørboe and Hansen 1993) when *Thalassiosira* sp. is aging. At this stage, the amount of EPS released is major compared with the exponential growth phase, probably because of an overflow mechanism to protect photosynthetic machinery at low nutrient concentrations (Smith and Underwood 2000). Furthermore, some diatoms are capable of heterotrophy, and the EPS released and broken down could be used as a carbohydrate source by reabsorption (Smith and Underwood 2000); however, it was not investigated here.

These results open new routes by which to understand the roles of EPS released by phytoplankton in the algae/bacteria associations in the planktonic environment. The potential of TEP and aggregate formation by diatoms and other phytoplanktonic groups should become a focus, as well as the changes in the bacterial community during the degradation period. Studies correlating bacterial dynamics to the composition and structure of polysaccharides released by phytoplankton, considering the advantages to the phytoplankton, would supply important information about the possible extracellular roles of these compounds.

We thank FAPESP (Processes 99/01353-5 and 99/07766-0) and PRONEX/CNPq for financing this project. We are indebted to Finn Tønnesen, University of Oslo-Norway, for performing the GC-MS analysis; Dr. Irineu Bianchini, Federal University of São Carlos-Brazil, for helping with the first-order decay equation; and the anonymous reviewers for useful suggestions.

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