Role of hydrophobic extracellular polysaccharide of *Aulacoseira granulata* (Bacillariophyceae) on aggregate formation in a turbulent and hypereutrophic reservoir

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Abstract

The highest density of aggregates (1.4×10^3 L^{-1}) in the hypereutrophic Barra Bonita Reservoir (mean depth of 10.2 m) coincided with the peak population density of the diatom *Aulacoseira granulata* (2.3×10^6 chains L^{-1}), which was present in 94% of the aggregates for 22 consecutive months. To investigate why this diatom, which produces very small amounts of free extracellular polysaccharide (EPS) and transparent exopolymer particles (TEP), occurs so frequently in natural aggregates, we carried out aggregation experiments with cultures of *A. granulata* with and without its free EPS and TEP. Stickiness (α) was higher in exponential than stationary growth, either with or without EPS, but the highest value was obtained in cultures without EPS (0.37). During the stationary growth phase, α decreased, both with and without EPS. Replacement of natural polymer carbohydrates by *A. granulata* free EPS (>14 kiloDalton [kD] < 0.4 μm), or adding cultured diatom to reservoir water, produced aggregates larger than the natural ones. The role of EPS in these aggregation characteristics was investigated by analyzing its monosaccharide composition. The proportion of terminal monosaccharides significant in the aggregation was 74.5%, 56.1% of them consisting of hydrophobic deoxy-sugars fucose and rhamnose which increase the stickiness. Early in the growth, only hydrophobic-adhering films are responsible for fast aggregation by chain-to-chain collision. With aging cells, aggregation was mainly by the trapping effect of free EPS and TEP, both formed by released adherent films. We hypothesize that aggregates have an important role in seeding *A. granulata* in environments with significant advective transport, such as reservoirs.

Aulacoseira granulata is a common diatom in eutrophic freshwater lakes and reservoirs around the world (Gomez et al. 1995; Hotzel and Croome 1996; Nogueira and Matsumura-Tundisi 1996). In fact, the filamentous *A. granulata* is one of the few diatom species that produce blooms in hypereutrophic freshwater environments. Routine observation of samples from Barra Bonita Reservoir showed that *A. granulata* is very often found in natural aggregates, even when it is not dominant as free chains in the phytoplankton community. We also observed that, compared to other diatoms, cultured *A. granulata* produces extremely low amounts of solute or colloidal extracellular polysaccharide, (<0.4 μm, denoted as free EPS in this text). Also, no particulate extracellular polysaccharide (>0.4 μm, denoted as TEP in this text) was detected in the early exponential growth phase. Even in the stationary growth phase free EPS and TEP production is much lower than the values usually reported for most marine diatom species in

research aggregation (Passow et al. 1994; Corzo et al. 2000). Even so, aggregation of *A. granulata* chains occurs in both phases, regardless of the presence or absence of TEP and free EPS. This has led to the supposition that in such species, during the early growth phases, the aggregation process could be relatively independent of free EPS and TEP concentrations. This process should be related to the chemical and or physical properties of the cell wall, besides the already well-known participation of TEP in late stages, as previously described for marine and lentic populations (Alldredge et al. 1993; Grossart et al. 1997).

It has been shown that turbulence, differential settling, and particle concentrations are the chief physical mechanisms in the coagulation of marine phytoplankton (Jackson 1990). However, published data indicate that other factors must be involved, in addressing aggregate formation. These include morphological and biological characteristics of the organisms, potential stickiness of the extracellular polysaccharides (EPS), and transparent exopolymer particles (TEP; Passow et al. 1994; Logan et al. 1995; Grossart et al. 1997). However, up until now, no data exist relating the chemical composition of EPS of a given diatom species with either its aggregation potential or aggregation mechanism. The effects of colloids (<0.4 μm, defined as dissolved in the oceanographic literature) on aggregation are usually very hard to quantify through models based on coagulation theory. This is because the chemical behavior of their constituents cannot be predicted, owing to the great

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complexity and great diversity of EPS in the environment (Wells and Goldberg 1994). Nevertheless, the chemical and conformational composition of colloids can determine specific biological characteristics in aggregation. In fact, as pointed out by Jackson (1995*b*) there are still many open questions about how mucilage affects the stickiness of diatoms.

As no data is available relating the chemical composition of a specific diatom EPS to its aggregation mechanisms, this paper focuses on this question. Furthermore, there are not published studies on aggregation, either in situ or in cultured freshwater diatoms from hypereutrophic environments. In fact, most of the literature concerning aggregate formation by diatoms focuses on marine environments (Passow et al. 1994; Mari 1999) and most of the available freshwater data refers to Lake Constance (Grossart and Simon 1993, 1998; Grossart et al. 1997). Lake Constance, however, is subalpine, deep, monomictic, and mesotrophic, whereas Barra Bonita Reservoir is tropical, shallow (mean depth: 10.2 m), hypereutrophic, and polymictic, where material resuspension has a key role in the distribution of particles in the water column (Dellamano-Oliveira et al. in press). The turbulence in Barra Bonita Reservoir is caused by wind, blowing along the reservoir's longitudinal axis and by a constant horizontal water flux entailed in hydroelectrical power generation. The latter imposes low water residence times: 37 days in summer and 137 in winter. High temperatures and absence of wind may occasionally cause short stratification periods (hours to few days).

The main objective of the present research was to obtain data on the biological characteristics of aggregation related to the chemical composition and forms of the EPS of *A. granulata*, in order to answer these questions: Is the chemical composition of its EPS related to the high occurrence of *A. granulata* in the aggregates of Barra Bonita Reservoir? Does *A. granulata* readily form aggregates, despite releasing little EPS and presenting low TEP, because of the chemical composition of its EPS? Are these *A. granulata* aggregates involved in seeding in environments that could cause their washout?

As far as we know, this study is the first to focus on the role of the chemical composition of EPS in the biological aspects of aggregate formation by a freshwater diatom in axenic cultures and aggregate formation in a freshwater hypereutrophic reservoir.

Methods

Barra Bonita Reservoir is located in the State of São Paulo (Brazil; 22°29′S, 48°34′W). It is formed by the confluence of Piracicaba and Tietê Rivers both of which are hypereutrophic and polluted by industrial and agricultural activity.

Aggregates at Barra Bonita Reservoir—Water samples from Barra Bonita Reservoir were obtained using Van Dorn samplers at 0-, 1-, 3-, 5-, and 10-m depth and at the bottom (18 m), once a month from Apr 02 to Jan 04. Large aggregates, obtained by filtering 1.0 liter of each sample through 75-μm-pore nylon screen, were collected with filtered reservoir water, and added to a 20-mL sedimentation chamber. Samples were allowed to sediment for 8 h, and then examined under an Axiovert inverted microscope (Zeiss). Aliquots of filtrate ($<$75 μm) were also analyzed for aggregates smaller than 75 μm. All aggregates, after being preserved with formalin and stained with Alcian Blue (8GX C.I.74240; Polysciences Inc.), were counted and measured under the microscope. Algal and cyanobacterial species associated with the aggregates were identified under a microscope.

Organism and culture conditions—*Aulacoseira granulata* var. *granulata* (Ehrenberg) Simonsen was isolated in 2002 from Barra Bonita Reservoir, and is presently kept as strain BB01 in the culture collection of the Botany Department at Federal University of São Carlos (World Data Center for Microorganisms No. 835). Axenic cultures were obtained by the method described in Vieira (1983). Experimental cultures were grown at pH 7 in whole culture Wright's Cryptophyte (WC) (Guillard and Lorenzen 1972) culture medium with the concentration of Na_2SiO_3 $9H_2O$ increased to 15×10^{-5} mol L^{-1}. Cultures were kept under the following controlled laboratory conditions: 100–120 μmol m^{-2} s^{-1} photon flux (Quantameter QSL-100; Biospherical Instruments); temperature of 23 $\pm$ 1°C; light:dark cycle of 12:12 h. After autoclaving, all culture media were aseptically filtered in precombusted glass fiber filters (GF-F; Whatman; 0.7-μm pore size) to eliminate possible precipitates. *A. granulata* cells were cultivated in 20-liter-capacity Pyrex carboys with 16 liters of culture which were gently shaken twice daily. Bacterial contamination was checked by inoculating an aliquot of the cultures into WC medium plus peptone, glucose, and yeast extract (150 mg L^{-1} each).

Microscopy—Cells, chains, and aggregates from cultures were counted under a light microscope with a Palmer–Maloney chamber. For qualitative evaluation purposes, aggregates, TEP in the medium, and extracellular polysaccharides adhering to the surface (adherent films) of *A. granulata* were negatively stained with India ink and or Alcian Blue and observed under a Zeiss Axioplan 2 microscope.

Aggregate formation and quantification—The aggregation methodology is described in Kiørboe and Hansen (1993); the horizontal Couette flocculator was constructed as described in van Duuren (1968). Samples (5 mL) were gently removed every 15 min and suspended particle concentrations counted in a Palmer–Maloney chamber. Three or four replicates were counted. Some drops of Indian ink added to the flocculator showed that particle distribution was homogeneous within $<$15 min. Cell aggregation was quantified using decay coefficients (k) obtained from suspended particle counts during the entire experimental period (150 min). To describe flocculated suspended particles, two mathematical models were used: one biphasic and one exponential. The exponential model proved more suitable, showing that particle aggregation followed an exponential pattern. The parameterizations were obtained by nonlinear regression (Levemberg–Marquardt iterative algorithm) as described in Press et al.

(1993) using the Eq. 1

$$C_t = C_0 + C'e^{-kt} \tag{1}$$

where C_t = suspended particle concentration at time t; C_0 = minimal theoretical limit of particle concentration; C' = particle concentration involved in aggregation; k = coefficient of particle decay during aggregation, and t = experimental time. Half-lives of aggregation were calculated as: $t_{1/2} = \ln (2)\, k^{-1}$. Values of stickiness ($\alpha$) of suspended cells were obtained by employing Eq. 2

$$\alpha = \text{slope } \pi \exp\left(\left[(1.5S^2) \times (d_1^2)^{-1}\right] \times (7.824\Phi G_m)^{-1}\right) \tag{2}$$

where slope = linear regression of ln (particle mL^{-1}) vs. time (s); S^2 = variance of the size distribution of individual particles (μm); d_1 = mean particle diameter (μm); Φ = initial particle concentration in volume fraction in mol L^{-1}, and G_m = mean shear rate, as described by Kiørboe et al. (1990) and Dam and Drapeau (1995).

Shear rates—The mean laminar shear rate G_m (s^{-1}) was calculated as described in van Duuren (1968). The effects of three shear rates (G_m = 3 s^{-1}, 10 s^{-1}, and 30 s^{-1}) on aggregation under culture conditions were determined as described above.

Influence of growth phase on aggregation—Aggregation experiments were conducted with cultured *A. granulata* chains, both with and without EPS. The experimental setup is shown in Fig. 1. The chains were sampled at different ages: 8, 18, 28, and 34th day of the culture, corresponding to the beginning, middle, and end of the exponential growth phase, and the beginning of the stationary phase, respectively. Exposure time for flocculation experiments was 150 min at a mean shear rate G_m = 3 s^{-1}. To set up the experiments with and without EPS, particle concentration in the flocculator was adjusted to 10^4 mL^{-1} chains, the mean concentration present in blooms at Barra Bonita Reservoir (Dellamano-Oliveira et al. in press). These chains were obtained from laboratory cultures by centrifugation (2,800 g). Because of the great difference in density between diatoms and TEP, during centrifuging these particles did not sediment. Therefore, "without EPS" also signifies "with neither free EPS nor TEP." For the experiment without EPS, the chains were washed twice with fresh WC medium before being transferred to the flocculator, which was filled with fresh WC medium. Absence of free EPS was verified by the method of Dubois et al. (1956). For the experiments with EPS, the chains were added to the flocculator filled with WC medium in which the diatom had been growing.

Addition of free EPS and chains of A. granulata *to reservoir water for aggregation*—The experimental setup is shown in Fig. 1. The free EPS (<0.4 μm; 2.16 mg L^{-1}) released by *A. granulata* on the 28th day of the culture was added to reservoir water previously filtered through a Xampler hollow fiber cartridge for tangential ultrafiltration (UFP-10-E-4A) with a nominal cutoff of 10 kiloDalton (kD) to remove natural particles and natural polysaccha-
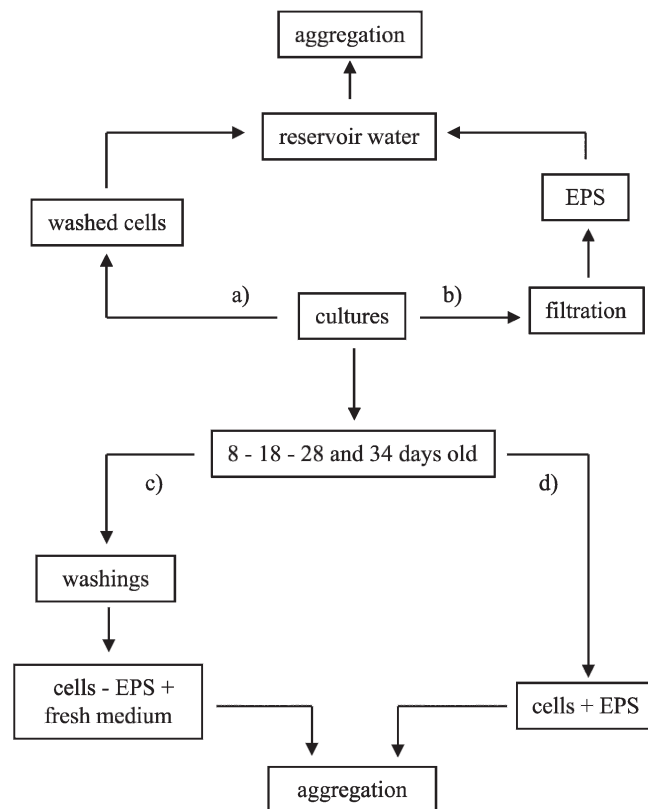

Fig. 1. Schematic diagram summarizing experiments carried out on aggregation of cultured *Aulacoseira granulata*. (a) Experiments with washed cells added to reservoir water at concentrations to simulate a bloom; (b) Addition of free EPS released by *Aulacoseira granulata*, replacing the natural polymeric carbohydrates in the reservoir water; (c) Effect of age on aggregation of diatom chains without released free EPS (from washed chains) of the diatom; (d) Effect of age on aggregation of diatom chains in the presence of its own released free EPS plus TEP.

rides (2.34 mg L^{-1}). Absence of polymeric carbohydrates in this medium, the EPS, and natural polysaccharides were determined by high-performance liquid chromatography and pulsed amperometric detection (HPLC-PAD) as described by Gremm and Kaplan (1997). Natural particles from 1.0 liter of reservoir water were obtained by tangential filtration (as above), further washed with ultrafiltered reservoir water, and added to the Couette flocculator to restore the natural particles, together with *A. granulata* EPS and ultrafiltered reservoir water. Retention efficiency, estimated by chlorophyll *a* (Talling and Driver 1963) before and after filtration, was close to 100%. Chains of *A. granulata* (10^4 chains mL^{-1}, mean of 4 cells $chain^{-1}$) were obtained from the same culture and washed by centrifuging (2,800 g). Absence of EPS was verified by the method of Dubois et al. (1956). The chains were then added to the reservoir water to simulate a bloom. The flocculation experiments were performed at a shear rate G_m = 3 s^{-1} for 150 min in duplicate. Natural population of *A. granulata* was determined by cell counts and reservoir water was also flocculated as a control.

Quantification of free EPS and TEP of diatom cultures—
At each age of the experimental cultures, cells were removed
by filtration through fiberglass filters (GF-F; Whatman).
After filtering in 0.4 μm (Isopore™), the free EPS released
into the media was determined by the methodology described
by Gremm and Kaplan (1997) for polymeric carbohydrates.
Amount of TEP in 100-mL samples taken at the various
growth phases of the cultures was measured as described by
Passow and Alldredge (1995).

*EPS isolation and characterization—*After 28 d of
growth, an axenic culture (8.0 liters) of *A. granulata* was
centrifuged (2,800 g) to remove the chains. The superna-
tant was set aside. The chains were washed under
agitation (60 min) with fresh WC medium (30°C), to
remove remaining free EPS, and retained on a glass-fiber
filter (GF-F; Whatman). The supernatant and filtrates
were pooled and concentrated in a rotary evaporator at
40°C. They were then dialyzed for 48 h against distilled
water. Dialysis tubes (Spectrapor™) with a 12–14-kD
molecular weight cutoff were used and some drops of
toluene were added to avoid bacterial contamination. The
dialyzed material was freeze-dried and stored at −8°C
under nitrogen. The freeze-dried material was further
purified as described in Giroldo et al. (2003), so as to
separate and detect possible EPS fractions. Monosaccha-
ride composition of the fractions was determined by gas
chromatography (Hewlett Packard [HP] 5890 Series II)
according to Barsett et al. (1992). Mannitol was used as
the internal standard. Monosaccharide linkages after
polymer methylation were determined by the method in
Kim and Carpita (1992); thereafter, analysis was per-
formed by gas chromatography and electron impact mass
spectrometry (HP selective detector 5970, linked to the
HP 5890 GC) of the derived and partially methylated
alditol acetates (Barsett et al. 1992; Samuelsen et al.
1995). Fractions with >5% of uronic acids were reduced
to the corresponding neutral sugars (Sims and Bacic
1995). Protein content in the EPS was determined by the
method of Lowry et al. (1951).

*Statistics—*Student *t*-test was used to contrast angular
coefficients (k) obtained from the linear regression plots of
particle decay, and α values. The same test was used to
contrast the mean value of aggregate formation and the
Nos. of particles aggregated. Mann–Whitney test was
applied to contrast the percent aggregation of separate
treatments. The Tukey test and analysis of variance
(ANOVA) were used to compare mean Nos. of particles
involved in aggregation at the various algal growth ages.

Results

*Aggregates in the reservoir—*The sampling methodology
may have some bias but it was the most appropriate for a
hypereutrophic environment in which the Secchi disk depth
is frequently 0.5 m, the photic zone ranges between 4 m
and 5 m and the water column is full of large colonies of
Microcystis aeruginosa and other cyanobacteria, that
inhibit diving and underwater photography or imaging in

the water column and clog the traps. The stability of the
aggregates facilitated the use of our methodologies.

Variation in the amount of aggregates (Fig. 2a) in the
entire water column of the reservoir from Apr 02 to Jan 04
matched the variation in concentration of *A. granulata*
chains in the water column (Fig. 2b). Aggregate abundanc-
es did not show a clearly defined distribution as a function
of depth; however, the highest concentrations occurred
between 5 m and the bottom (Fig. 2a). Aggregate areas
varied between 156 $\pm$ 12 and 79,750 $\pm$ 3,270 μm², with a
mean value of 8,243 $\pm$ 429 μm². It is noteworthy that the
maximum aggregate concentrations occurred simulta-
neously with or immediately after *A. granulata* blooms
and never during or after the maximum density of
cyanobacteria (Fig. 2c). This indicates that *A. granulata*
was connected with higher aggregate densities over the
sampling period.

Aggregates with a murky appearance due to the great
quantity of detritus were of a sufficient stability to permit
handling. Large Nos. of *Microcystis* sp. free cells (not
counted) were always present in the aggregates (Fig. 3a).
The presence of other cyanobacteria with aerotopes
(*Anabaena spiroides*, *Planktothrix tropicalis*, and *Cylindro-
spermopsis raciborskii*) was also frequent in the aggregates.
In addition, the presence of planktonic diatoms such as
Cyclotella sp., *Aulacoseira ambigua*, *Rhizosolenia* sp., and
some Penales diatoms was also constant (Fig. 3b), although
their frequency showed no correlation with aggregate
concentration. Many other particles, including pieces of
cladocerans, bacteria, debris, and other unidentified
particles made up part of the aggregates. *A. granulata*
was present in 94% of all aggregates formed over the
sampling period, including those in the winter blooms of
cyanobacteria, even when the diatom was not dominant in
the phytoplankton community (e.g., Feb 03; Fig. 2b).

*Characterization of EPS—*Molecular weight was esti-
mated as $\geq$2,000 kD by comparing its void volume with
that of Blue Dextran polymer in Sepharose CL 6B gel
filtration. Analysis (Giroldo et al. 2003) showed that EPS
yielded only one major fraction. Small amounts of a neutral
fraction (<0.01%, w : w) were also detected. This probably
consisted of intracellular compounds released after cell
death and or extracted during washings of the cells
(Chiovitti et al. 2004) and not part of the EPS.

Rhamnose (31.3%), xylose (24.5%), glucuronic acid (13.2%),
and fucose (7.8%) were the main EPS monosaccharides. Small
amounts of six other monosaccharides were also detected
(Table 1). Protein content was 6%. The polysaccharide, a
complex branched heteropolysaccharide, was essentially com-
posed of 1,4 linked terminal xylose; 1,2,4 linked rhamnose and
1,2,4 glucuronic acid (Table 2). This polysaccharide has a
central branched portion composed of 1,2,4 linked rhamnose
and 1,2,4 linked glucuronic acid, besides linear chains made up
principally of 1,4 linked xylose and minor amounts of 1,2
linked rhamnose, 1,3 linked galactose, mannose, and galac-
turonic acid. Among the 27.1% monosaccharides that were
terminal units, 56.1% was represented by the deoxy sugars
fucose and rhamnose, with recognized hydrophobic properties.
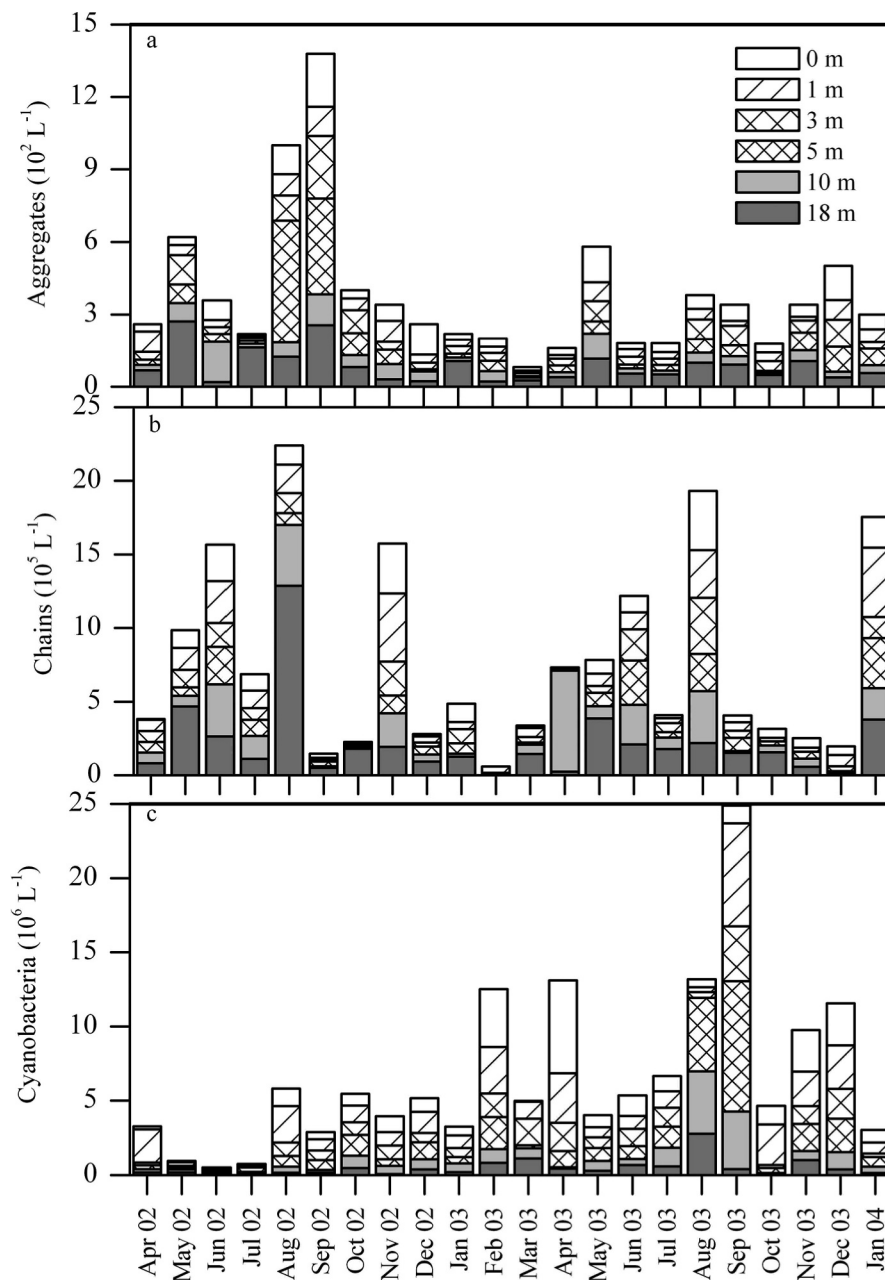Including the terminal units of glucuronic acid, whose reactivity

Fig. 2. (a) Aggregates density (L^{-1}), (b) abundance of *Aulacoseira granulata* (chains L^{-1}), and (c) abundance of cyanobacteria (organisms L^{-1}, including isolated cells, filaments and colonies) in water column. Coefficient of variation: (a) = 5%, n = 3; (b) = 29%, n = 3; (c) = 12%, n = 3.

is also recognizable, the percentage of terminal monosaccharides significant in the aggregation process reaches 74.5%. N-acetyl-galactosamine (0.7%) and N-acetyl-glucosamine (3.2%) were absent after methylation, either due to their degradation during the process or to insufficient hydrolysis.

Addition of A. granulata *EPS to the reservoir water*—The No. of aggregates formed when the natural polymeric carbohydrates from the reservoir were replaced by free EPS from cultured *A. granulata* (BB+EPS) was lower than that formed in the control (BB water). However, the aggregates formed in BB+EPS were larger; the No. of aggregates with

areas larger than 30,000 μm^2 was ~5 times higher than that observed in BB water (Table 3). The intermediate size classes also showed significantly higher relative abundance in the BB+EPS (Table 3). The BB+EPS aggregates were formed by a greater diversity of cyanobacteria, diatoms, and other microalgae, besides detritus entangled in a mucilaginous matrix, forming flocs of a less firm consistency that those obtained in BB water.

Addition of A. granulata *cells to the reservoir water*—The aggregates formed when cells were added to reservoir water (BB+Cells) were larger but very similar in composition and
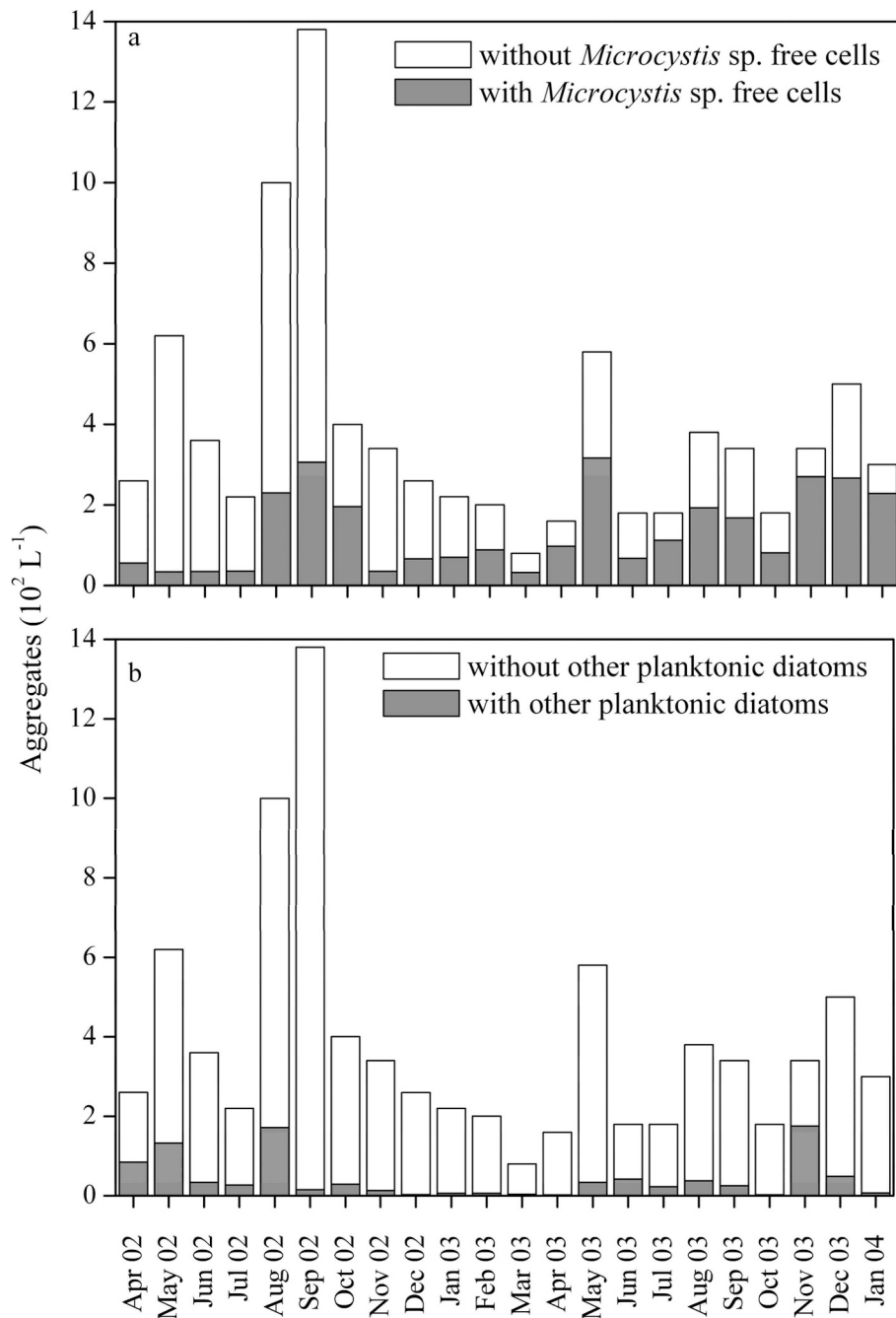
Fig. 3.   (a) Density of aggregates (L^{-1}) with and without free cells of *Microcystis* sp., and (b) with and without planktonic diatoms other than *Aulacoseira granulata*.

appearance to those formed in BB water. They were composed mainly of *A. granulata* chains, to which other particles, including debris, cyanobacteria, diatoms, and other micro-algae adhered, with very little colloidal material (Table 3).

Growth pattern of A. granulata—The experimental cultures of *A. granulata* followed the typical growth curve expected in batch cultures (Fig. 4). Under the conditions used and in batch culture, *A. granulata* does not produce high population densities, which usually range between 1.2 $\times$ 10^5 and 1.6 $\times$ 10^5 cells mL^{-1}.

Influence of shear rates on aggregate formation—Chain aggregation was dependent on shear rates (Fig. 5), with values of stickiness decreasing with increasing of shear rate. At $G_m = 3$ s^{-1}, $\alpha = 0.29$ was statistically similar to that obtained at $G_m = 10$ s^{-1} (0.22), but at $G_m = 30$ s^{-1}, $\alpha = 0.10$ was significantly smaller.

Aggregates formation in different growth phases—No significant chain aggregation was observed on the 8th day of experiments *with* or *without* EPS (Fig. 6). At this age, free EPS concentration in the media was very low (Fig. 7), and

Table 1. Monosaccharide composition (% of total carbohydrate) determined by gas chromatography of trimethysilylated derivatives of methyl-glycosides obtained by methanolysis of a single fraction of EPS released by *Aulacoseira granulata*.

Monosaccharide	%
Rhamnose	31.3
Xylose	24.5
Glucuronic acid	13.2
Fucose	7.8
Galactose	7.0
Galacturonic acid	6.2
Mannose	5.4
N-acetyl-glucosamine	3.2
N-acetyl-galactosamine	0.7
Glucose	0.7

neither were adherent films observed covering the chains nor was TEP detected in the media (Fig. 8). However, aggregate formation did occur on the 18, 28, and 34th day in both experiments, when polysaccharide adhering to the chains

Table 2. Glycosidic linkages of the monosaccharides present in a single fraction of EPS from *Aulacoseira granulata* determined by gas chromatography and electron impact mass spectrometry of the corresponding partly methylated alditol acetates. The fraction of each linkage in the same monosaccharide (% M) and in the total EPS (% EPS) are reported. T = terminal position; m.u. = mass unit.

	Linkages	Fragments (m.u.)	% M	% EPS
Rhamnose	T	118,131,162,175	32.8	10.3
	1,2	131,190	18.8	5.8
	1,3	118,131,234	11.6	3.7
	1,2,4	190,203	31.6	9.8
	1,3,4	118,275	5.2	1.7
	Total		100	31.3
Fucose	T	118,131,162,175	62.8	4.9
	1,2,3	131,262	37.2	2.9
	Total		100	7.8
Xylose	T	117,118,161,162	11.0	2.7
	1,4	118,189	89.0	21.8
	Total		100	24.5
Mannose	T	45,118,161,162,205	15.3	0.8
	1,2	45,161,190,205	15.6	0.8
	1,3	45,118,161,234,275	53.6	3.0
	1,4	45,118,162,233	15.5	0.8
	Total		100	5.4
Galactose	T	45,118,161,162,205	26.2	1.8
	1,3	45,118,161,234,275	44.6	3.2
	1,4	45,118,162,233	14.2	1.0
	1,6	118,162,189,233	15.0	1.0
	Total		100	7.0
Glucose	T	45,118,161,162,205	31.1	0.2
	1,2	45,161,190,205	68.9	0.5
	Total		100	0.7
Glucuronic acid	T	47,118,162,163,207	38.0	5.0
	1,2,4	47,162,263	62.0	8.2
	Total		100	13.2
Galacturonic acid	T	47,118,162,163,207	22.4	1.4
	1,4	47,118,162,235	77.6	4.8
	Total		100	6.2

Table 3. Size of aggregates (%) formed in the water of Barra Bonita Reservoir (BB water = control), in BB water plus EPS from *Aulacoseira granulata* cultures (BB+EPS), and in BB water plus cultured cells of the diatom (BB+Cells). Time of flocculation = 180 min: G_m = 3 s^{-1}. Cells i and Cells f = initial and final cell densities (cells mL^{-1}). Cells % = percentage of cells aggregated at end of experiments. In the intermediate size classes, aggregates presented a significantly higher relative abundance in the BB+EPS (Mann–Whitney test: p = 0.0317 between 10,000 and 20,000 μm^2, and p = 0.0159 between 20,000 and 30,000 μm^2. Mean ± SD, n = 6.

	Percentage of each size class		
Size class (μm^2)	BB water	BB+EPS	BB+Cells
<10,000	84.4±4.7	61.1±7.3	64.2±3.7
10,000–20,000	11.3±2.8	19.5±5.3	20.3±5.0
20,000–30,000	2.5±1.1	8.7±4.3	7.2±2.1
>30,000	2.0±1.95	10.8±3.7	10.3±4.4
Total aggregates L^{-1}	20,701±2,499	15,605±2,030	22,731±2,499
	Cell densities		
Cells i	339±65	414±52	39,738±3,517
Cells f	212±63	137±41	19,238±1,615
Cells %	37.5%	67%	51.6%

could be observed by staining with Alcian Blue or negatively stained with Indian ink on the 18 and 28th days. The value of α was higher on the 28th in both experiments (Table 4).

In cultures *without* EPS, where the polysaccharide films coating the chains were presumably the only sticky material, chain-to-chain collision was the dominant, if not the only, process of aggregation. The increase of α in the exponential growth phase coincided with the appearance of adherent films on the chains, after the 18th day. The aggregates were mainly formed of 2–8 chains and were
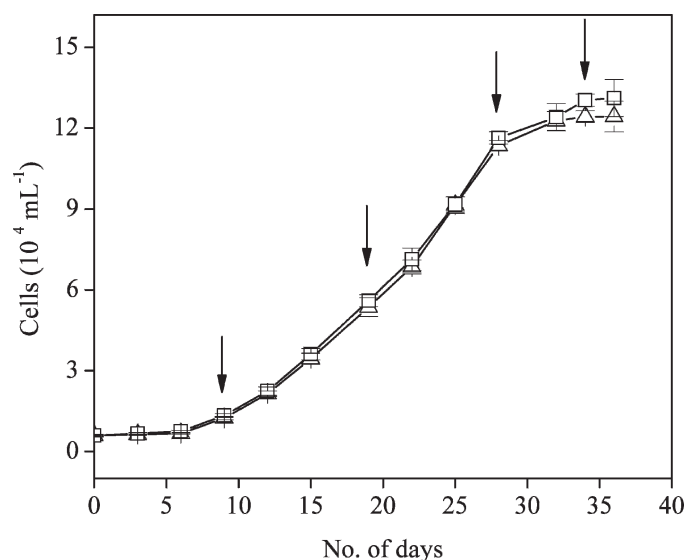

Fig. 4. Growth curve of *Aulacoseira granulata* in culture. Arrows correspond to the sampling for flocculation experiments. Triangles and squares indicate duplicate cultures. Intrinsic rate of increase (r) = 0.12; doubling time (T_2 = 0.6931 r^{-1}) = 6.03 d. Error bars represent standard deviation, n = 6.
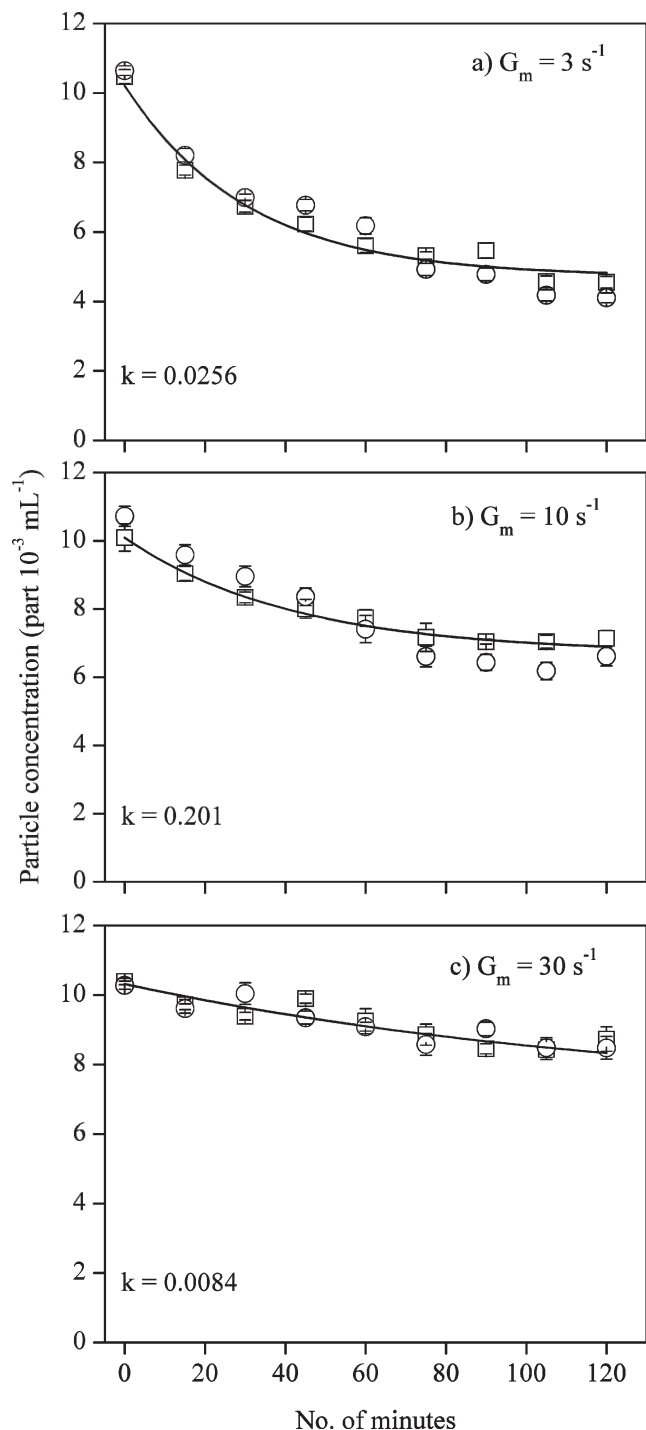
Fig. 5. Aggregation of particles in suspension (chains of *Aulacoseira granulata*) in the Couette device, under different shear rates (G_m = [a] 3, [b] 10, and [c] 30 s^{-1}), as a function of time of flocculation. k = coefficient of particle decay. Circles and squares indicate duplicate experiments. Error bars represent the standard error of the mean, n = 3. α is similar at 3 and 10 s^{-1} (Student *t*-test [df = 3]: p = 0.2902).

quite stable enough to be handled. The marked decrease of α on the 34th day in the cultures *without* EPS was apparently due to the release of most of the adherent films, which were washed out as free EPS.

In cultures *with* EPS the value of α increased until the 28th day, with the production of adherent films, free EPS, and TEP (Table 4). In the exponential growth phase, the coagulation could happen by chain-to-chain collision, by entanglement of the chains by free EPS, and TEP could also participate in the aggregation by collision with chains, despite its low concentration. Since a great part of the polysaccharide from adherent films was released to the medium, by the 34th day coagulation was dominated by the free EPS trapping effect and or chain-to-TEP collision. At this age, the aggregates were floccose, composed of a mucilaginous matrix trapping small aggregates and isolated chains.

Discussion

The polysaccharide—The method used to obtain EPS released into the media may have also extracted some adherent films. Even so, our results suggest that the adherent films should have a composition similar to, if not the same as, the released EPS. The small concentration of EPS during the exponential phase and its rise from the 28th day suggest that adherent films are the main source of free EPS.

The effect of EPS on aggregation depends on polysaccharide reactivity (Mopper et al. 1995). The methylated sugars fucose and rhamnose, which are known for their hydrophobic properties due to the methyl group at carbon 6, represent 56.1% of the terminal units of the EPS of *A. granulata*. In addition, the presence of uronic acids in the EPS confers a negative charge to the polysaccharide and increases its reactivity (Decho 1990). Besides this, uronic acid moieties in polysaccharides may form cationic bridges with metals commonly found in natural waters and culture media, mainly Ca^{2+}, Na^+, Mg^{2+}, giving a fibrilar and colloidal structure to the EPS, which increases aggregation by particle capture (Leppard 1995; Passow 2002). These characteristics help to explain important features of this system. First, easy aggregate formation in *A. granulata* cultures by chain-to-chain collision; second, the trapping effects; third, the enlargement of aggregates when EPS, either free or adhering (as film) to chains of *A. granulata*, is added to reservoir water; and, fourth, the high frequency of *A. granulata* in the aggregates found in Barra Bonita Reservoir.

However, in nature, several factors may interfere with the polysaccharide properties. For example, bacterial activity may act upon and alter its stickiness by selective EPS degradation, which may increase or decrease the relative concentration of its hydrophobic monosaccharides (Giroldo et al. 2003). The presence of bacteria was positively detected only 7–14 d (in a few samples) after the experiments had finished. This means that contamination probably occurred because of a small No. of laboratory bacteria. However, these would not have had enough time to cause measurable effects (Middelboe and Søndergaard 1993; Giroldo et al. 2003).

Shear rate—The stickiness of the *A. granulata* is dependent on shear rate, high shear rates either reducing aggregate formation or leading to disintegration of previously formed aggregates, as happened at G_m =
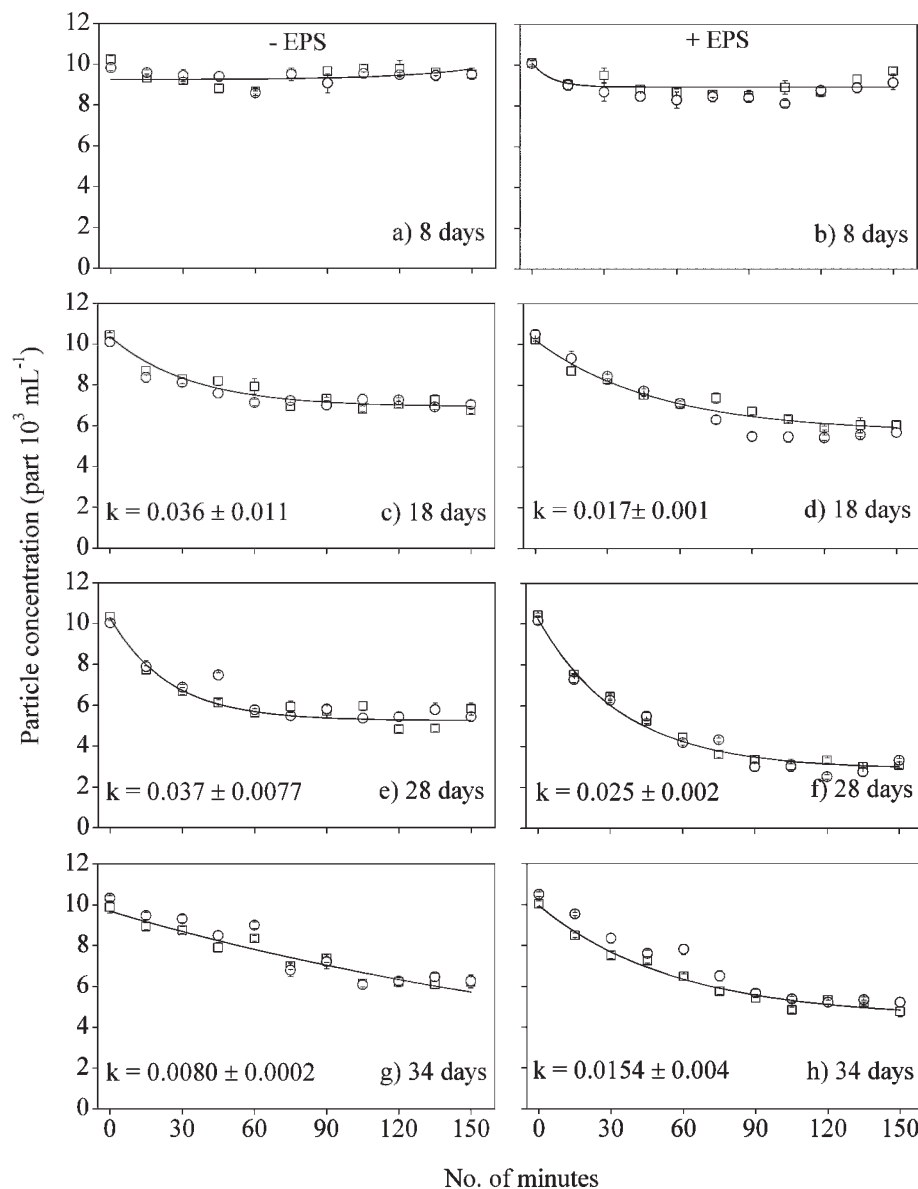
Fig. 6.   Aggregation of particles in suspension (chains of *Aulacoseira granulata*) on the 8 (a, b), 18 (c, d), 28 (e, f), and 34th (g, h) day in cultures without (−EPS) and with EPS (+EPS). $k =$ coefficient of particle decay. Symbols indicate duplicate cultures. $G_m = 3$ s^{-1}; $t = 150$ min. Error bars represent the standard error of the mean, $n = 3$.

30 s^{-1}. Similar results were obtained by Kiørboe and Hansen (1993) for *Skeletonema costatum* cultures. Shear rate at epilimnion was estimated by energy dissipation rate (ε) based on wind velocity at 2.0 m above surface ($W_{max} = 8.78$ ms^{-1}). During 50% of sampling period $\varepsilon = 1.3 \times 10^{-5}$ m$^2 \times$ s^{-3}, 3.2×10^{-5} m$^2 \times$ s^{-3} during 67%, and 1.3×10^{-4} m$^2 \times$ s^{-3} during 10% of the time which allowed us to estimate the G_m <3.5 s^{-1}, <5.6 s^{-1}, and >11.5 s^{-1} respectively (MacKenzie and Leggett 1993). According to our results, even an increase in the shear rate up to 10 s^{-1}, would not alter α value significantly.

Effect of age on aggregation—A summary of the described aggregation mechanisms is sketched in Fig. 9.

The present results suggest that the effect of age on aggregation of *A. granulata* is related to the chemical composition of EPS, its production rate profile, and its forms (e.g., adhered to the chains as thin film, free in the medium as solute or colloidal mucilage [free EPS], or particulate as TEP). The importance of EPS in aggregate formation is illustrated by the absence of aggregation on 8th day in cultures *with* and *without EPS*, when no adherent films were detected, and also by the lowest values of stickiness at 34th day in *without EPS* where the released adherent film was washed out (*see* below). Our observations confirm that in the exponential growth phase the mucilage coating the cells was similar to a thin film (Hoagland et al. 1993) occasionally with adhered "clouds"
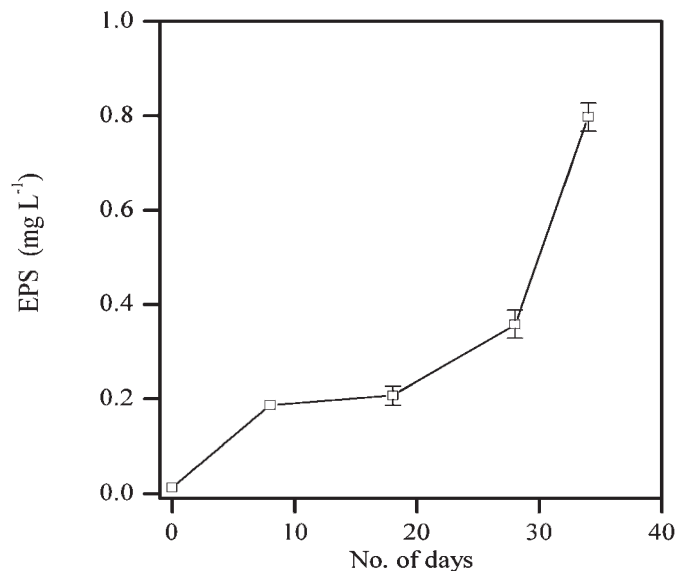
Fig. 7.   Concentration (mg L^{-1}) of free extracellular poly-saccharide (free EPS) in the culture medium as a function of culture age. Error bars represent the standard deviation of the mean, $n = 3$.

on some cells. Release of these films began to increase at the end of the exponential growth phase, as proposed by Decho (1990). This explains low free EPS and undetectable TEP in the culture media up to the 28th day.

The highest α values and the smallest half-lives obtained for cultures *without EPS* in the exponential phase lead us to conclude that, in the field, growing chains of *A. granulata* coated with hydrophobic EPS quickly aggregate by chain-to-chain collision, even in the theoretical absence of any free EPS or TEP. The results obtained in cultures *with EPS*, which is assumed to occur in the field, showed that coagulation could be dominated by chain-to-chain collision early in blooms. At the end of blooms, the coagulation would be dominated by the trapping effect of free EPS
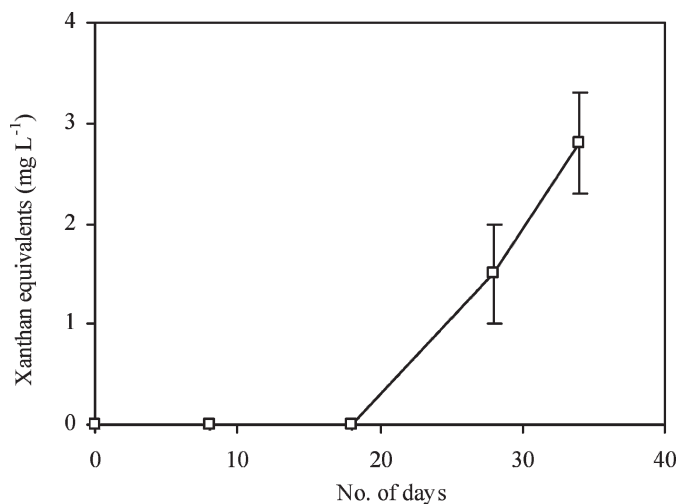

Fig. 8.   Concentration of TEP (including the adhering films), in xanthan equivalents (mg L^{-1}), as a function of culture age. Error bars represent the standard error of the mean, $n = 3$.

Table 4.   Percentage of aggregated particles (%), stickiness (α), half-lives ($\ln[2]k^{-1}$) for aggregation of particles and No. of particles aggregated. Note the highest No. of particles aggregated on the 28th day (ANOVA $p = 0.010$; Tukey test: $8 < 18 = 34 < 28$th day). The α values are similar on the 18 and 28th day in $-$EPS (Student *t*-test (df = 3); $p = 0.8914$). Mean $\pm$ SD; $n = 3$.

Exper.	Age (days)	%	α	Half-life (min)	Particles aggregated
	8	–	–	–	
$-$EPS	18	32.8	0.36	19.3	3,373$\pm$310
	28	44.6	0.37	18.8	4,534$\pm$46
	34	38.1	0.08	86.8	3,875$\pm$225
	8	–	–	–	
$+$EPS	18	43.4	0.17	40.8	4,495$\pm$328
	28	68.7	0.25	27.8	7,072$\pm$248
	34	51.5	0.15	45.1	5,292$\pm$8

(with possible participation of other colloids in the water) and or chain-to-TEP collision, since the adhesion of chain-to-chain is markedly reduced. In an environment with constant turbulence, this type of aggregate is subject to disruption; this may be the reason for the absence of large aggregates in Barra Bonita.

The smaller value of α on the 18 and 28th days *with* EPS must be discussed. First, we must point out that the effect of free EPS ($<0.4 \mu m$) was not taken into account in the equations used to calculate stickiness, as usual in experiments of aggregation described in the literature. The pattern of aggregation in cultures both *with* and *without* EPS on 18th day was very similar to that found by Gibbs (1983) for coated and uncoated particles (his Fig. 1). At this age, the concentration of released EPS was not high, and there were enough adherent films to cause chain-to-chain aggregation. However, even at low concentrations the released EPS should be enough to produce an effect similar to that described by Gibbs (1983): natural coated particles coagulated significantly more slowly than particles with the coatings removed (*see* also Leppard 1995). Decho (1990) suggests that a dense polysaccharide sheath deposited on diatom cells in the exponential growth phase would be transformed into a less dense one in the stationary growth phase; this would decrease aggregation. If this supposition is right, the free EPS released into the medium is less hydrophobic than the adherent films, not necessarily through changes in its chemical composition: the releasing of adherent films in itself could change their conformation, (e.g., by modifying the position of hydrophobic terminal units formerly exposed to the outside [e.g., turning them towards other molecules or to cell surfaces]; Rees 1977). Or, theoretically, after EPS release, the uronic acids (6.4% of terminal units) could be more available for salt formation by binding cations, altering the polymer conformation. It could then act as an organic coating "net," stabilizing the particles by, for example, wrapping the chains and, consequently, diminishing either the hydrophobic surfaces of the chains or the contact adherent film to adherent film (Leppard 1995). Any of these events would decrease α and, since the percentage of aggregated particles in *with* and *without* EPS cultures are close on the 18th day, consequent-
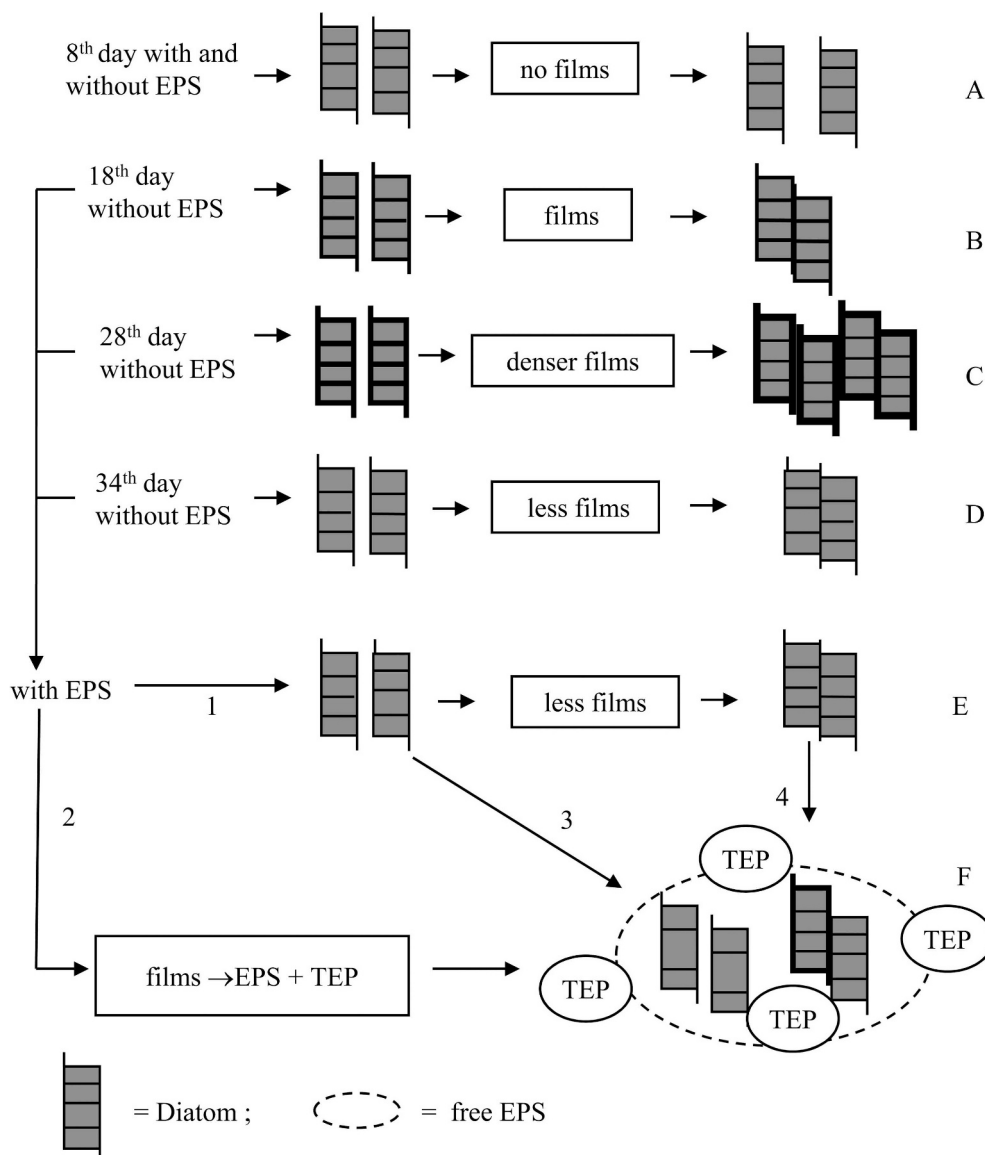
Fig. 9. Diagram demonstrating the proposed mechanisms of aggregation at different ages of cultures and forms of EPS. A) There is no aggregation because sticky films have not yet formed. B) Formation of sticky films allows aggregation by chain-to-chain collision. C) Increased formation of EPS for sticky films increases the stickiness and the rate of aggregation by chain-to-chain collision. D) Release of EPS forming the adherent films leads to their being washed out, sharply reducing aggregation. E) In culture experiments with EPS, on the 18 and 28th days, aggregates are formed by chain-to-chain collision at a reduced rate because the free EPS reduces chain-to-chain collision. 1) With EPS, on 18 and 28th day some aggregation occurs by chain-to-chain collision because there are adherent films yet; hence, the α values are higher than on the 34th day. 2) The extracellular polysaccharide forming the films is released to form free EPS and TEP. 3) Isolated chains are entangled by free EPS and/or TEP. 4) Small aggregates composed of few chains are also entangled by free EPS together with TEP, forming floccose aggregates.

ly also would increase half-lives. These events may also occur on the 28th day, but in addition α may have been underestimated, since the proportion of aggregated chains in *with* EPS cultures was higher than in *without* EPS with higher α. Neither the No. of TEP particles nor the free EPS was considered in the calculation of α and the aggregates in *with* EPS were larger than in *without* EPS, so that α could be underestimated (Engel 2000). Also, the aggregates

formed mainly by means of the free EPS trapping effect and TEP are floccose and large and are susceptible to disruption. This would cause underestimation of stickiness, since desegregation in the Couette flocculator does not remove particles, which return to aggregation (Jackson 1995*a*; Waite et al. 1997). On the 34th day *with* EPS, the α value was higher than that *without*, because in the latter there was neither film nor EPS. The release of the adherent

films at the stationary phase could offer a reasonable explanation for the high aggregation half-lives, the effects being equivalent to that of a substance that limits aggregation, as found in *Skeletonema costatum* (Kiørboe and Hansen 1993). The underlying reason that adherent films are released is still to be elucidated.

Concentration of TEP in Barra Bonita Reservoir is high, owing to the dominance of cyanobacteria such as *Anabaena spiroides*, which have thick capsules of acidic polysaccharide. Fragmentation of such capsules liberates considerable amounts of presumed nonfractal TEP (A. A. H. Vieira and T. B. Bittar unpubl.). Such TEP could have cooperated in the aggregation of *A. granulata* in the reservoir (Alldredge et al. 1993; Logan et al. 1995). However, the highest concentrations of aggregates and cyanobacteria never matched. Similar results were reported by Grossart et al. (1997) in Lake Constance.

Ecological consequences—Barra Bonita Reservoir is a hypereutrophic environment (Dellamano-Oliveira et al. in press), rarely stratified and remarkably shallow, which would lessen the importance of the ecological function of aggregates sinking to reach nutrients, as described by Smetacek (1985) for marine environments. The frequent occurrence of free cells of *Microcystis* sp. and planktonic diatoms other than *A. granulata* in aggregates indicates that these particles should have an important role in the ecology of reservoirs, related to the sinking of small cells with positive buoyancy and in a possible occurrence of the mutual flocculation mechanism by which some diatoms may disappear from the environment (Hansen et al. 1995). However, here the discussion will be restricted to the possible ecological role of the aggregates in seeding *A. granulata*.

The presence of *A. granulata* in the majority of aggregates from Barra Bonita Reservoir, and the coagulation of 51% of cultured chains added to reservoir water (simulating a bloom), suggest a possible key role for aggregation in the life cycle of this diatom, which produces no spores.

Early on the blooms part of the diatom population would rapidly aggregate (low half-life), forming initially small aggregates with growing cells. These would rise in density by the aggregation of other particles, enough to allow them to sink or to avoid being washed from the reservoir, even in a polymictic environment, thus keeping part of the population for seeding by easy resuspension due to the turbulence and the very rare occurrence of thermoclines. Later, the aging population may also aggregate, but with greater half-lives. The constant resuspension may explain the occurrence of the diatom around the year in the plankton, not only as free chains but also as aggregates. This would be a mechanism by which *A. granulata*, with a doubling time close to 6 d, is present not only in great reservoirs such as Barra Bonita but also in eutrophic, smaller and shallower water bodies like Monjolinho Reservoir (São Carlos, São Paulo State, Brazil). In this reservoir, the diatom occurs around the year even when the hydraulic residence time is 2.1 d (Nogueira and Matsumura-Tundisi 1996). The aggregates could have a role similar to that played by statospores in

the perennation strategies of some chrysophycean species (which, relevantly, also occur in this reservoir): they are formed irrespective of prevailing environmental conditions (e.g., nutrients and temperature) to guarantee the seeding for future populations in such environments (Sandgren 1988).

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