Aggregate formation in axenic and microbial co-inoculated batch cultures of Aulacoseira granulata (Bacillariophyceae).

VIEIRA[1], A.A.H., GIROLDO[2], D. & ORTOLAN[2], P.I.C.

[1]Laboratório de Ficologia, Departamento de Botânica, Universidade Federal de São Carlos, Rodovia Washington Luis Km 235, 13565-905, São Carlos, SP, Brasil. E-mail ahvieira@power.ufscar.br
[2]Departamento de Ciências Morfobiológicas, Fundação Universidade Federal do Rio Grande, Avenida Italia, Km 8, 96201-900, Rio Grande, RS, Brasil.

ABSTRACT: Aggregate formation in axenic and microbial co-inoculated batch cultures of Aulacoseira granulata (Bacillariophyceae). Aggregate formation involving diatoms is a well-known process, especially in the marine environment. The aim of this paper was to observe natural aggregates formation in axenic and microbial co-inoculated cultures of the freshwater diatom Aulacoseira granulata. The growth of bacteria and A. granulata were also monitored, as were qualitative alterations in the composition of the extracellular polysaccharide (EPS) produced by A. granulata in both axenic and microbial co-inoculated cultures. Aggregates were naturally formed in both conditions and our results suggested that the aggregation process increased significantly in the presence of a microbial community from Barra Bonita Reservoir. A. granulata biomass formation also significantly increased along bacterial growth and the EPS composition found in the microbial co-inoculated cultures was different to that of the axenic cultures. Rhamnose proportion increased around 50 % in microbial co-inoculated cultures, probably due to bacterial activity, increasing the hydrophobic properties of the EPS. The aggregation increase in the microbial co-inoculated cultures was correlated to the enhancement of the EPS hydrophobic nature, as well as to the biomass increase of A. granulata.
Key-words: Aulacoseira granulata, aggregates, extracellular polysaccharide, microbial degradation, bacterial growth.

RESUMO: Formação de agregados em culturas de Aulacoseira granulata (Bacillariophyceae) axênicas e inoculadas com bactérias. A formação de agregados envolvendo diatomáceas é já um processo bem conhecido, especialmente em ambientes marinhos. O objetivo deste trabalho foi observar a formação espontânea de agregados em culturas de Aulacoseira granulata axênicas e inoculadas com bactérias. O crescimento de bactérias e de A. granulata foi monitorado bem como as alterações qualitativas, causadas pela atividade bacteriana, na composição do polissacarídeo extracelular (EPS) liberado pela diatomácea em ambas as culturas. A formação de agregados ocorreu em ambas as culturas, mas os resultados sugerem que a agregação aumenta significantemente na presença de bactérias de populações oriundas do reservatório de Barra Bonita, também o local de origem da diatomácea. A biomassa de A. granulata aumentou significantemente na presença de bactérias e a composição do EPS nas culturas contaminadas foi diferente daquele das culturas axênicas. A atividade bacteriana aumentou a porcentagem do monossacarídeo Ramnose em cerca de 50 % nas culturas contaminadas, aumentando as propriedades hidrofóbicas do EPS. Assumimos que o aumento da hidrofobicidade bem como o aumento da biomassa de A. granulata nas culturas contaminadas foram os fatores responsáveis pelo aumento da agregação dos filamentos de A. granulata nas culturas contaminadas.
Palavras-chave: Aulacoseira granulata, agregados, polissacarídeo extracelular, degradação microbiana, crescimento bacteriano.

Introduction

Organic aggregates are extensively studied, mainly in seawater, because of their ecological significance for aquatic environments, both fresh and seawater.

They are involved in processes such as microbial loop, sinking flows of both organic and inorganic materials and coagulation of phytoplanktonic blooms (Kjørboe & Hansen, 1993; Leppard, 1995; Grossart & Simon, 1998). Microbial activity

in both macroaggregates (›500mm), and microaggregates (‹500 mm) is a remarkable characteristic, and they have been identified as hot spots of microbial decomposition of organic matter (Simon et al., 2002). TEP (transparent exopolimeric particles) also play a significant role in aquatic ecology and they are considered an important stage in the formation of macro and microaggregates (Alldredge et al., 1993).

The constitution of aggregates varies greatly depending on the environment and system (Simon et al., 2002). However, in general, they consist of a polysaccharide matrix and particles both organic, such as phytoplanktonic cells, zooplankton molts and carcasses, bacteria and remains of cell walls and inorganic, such as sand, clay and silica crystals. The polysaccharide matrix could originate from both phytoplankton (Passow, 2002) and bacterioplankton (Stoderegger & Herndl, 1998) exopolysaccharides (EPS), although the first is considered the most significant because of the large amounts of EPS produced by dense phytoplanktonic blooms (Stoderegger & Herndl, 1998). Large macroaggregates reaching several meters in length have regularly been found after diatom blooms in the Adriatic Sea and this has been related to the release of EPS by these organisms (Alcoverro et al., 2000).

The influence of phytoplanktonic cells and EPS in the formation of aggregates under controlled conditions in rolling tanks and coagulators has extensively been studied (Shanks & Edmondson, 1989; Kjørboe & Hansen, 1993; Agis et al., 1997). On the other hand, data on natural aggregate formation in freshwater phytoplanktonic cultures are unknown. Furthermore, the influence of bacterial activity on aggregate formation has scarcely been studied (Giroldo et al., 2003). In this study, measurements were taken of the aggregate formation in axenic and microbial co-inoculated batch cultures of Aulacoseira granulata, a freshwater diatom frequently dominant in the plankton of eutrophic reservoirs. The composition of the EPS produced by both axenic and contaminated cultures was analyzed, in order to check alterations in the EPS caused by selective bacterial activity.

Material and methods

Aulacoseira granulata var granulata (Ehrenberg) and the surrounding heterotrophic microbial community were collected in the euphotic zone (0 – 5 m, determined by a Spherical Quantum Sensor LI-193SA and a LI-250 Light Meter LiCor™ Lincoln, Nebraska) of the Barra Bonita reservoir, located on the Tietê River, São Paulo, Brazil (22° 29' S and 48° 34' W). A. granulata was isolated directly by microscopy and axenic cultures were obtained by washing with Dakin solution (Vieira, 1983). The culture medium was complete WC (Guillard & Lorenzen, 1972) modified by the addition of 150 mM Na_2SiO_3 . 9 H_2O (WC-Si plus), and the cultures were kept under 100 mmol photons m^{-2} s^{-1} (Photosynthetically Active Radiation) at 22 ± 1° C and in a dark:light cycle 12:12 h. Tests to check for bacterial contamination in the axenic cultures were performed regularly with WC-Si plus medium modified by the addition of glucose and peptone (250 mg L^{-1} of each one).

The microbial inoculum was aseptically filtered at low vacuum pressure (10 mm Hg) through calcinated glass-fiber pre-filters AP-20 (Millipore Dublin, California), with approximately 10 mM of pore size, to remove particles, and zoo and phytoplankton.

A dense axenic inoculum of A. granulata (1 L, 10^5 cell.mL^{-1}) in the exponential growth phase (15 days) was inoculated in 15 L of fresh WC-Si plus and left for three days in the conditions described above. Afterwards, the 15 liters culture were divided in four fractions of 3.5 L, where two were inoculated with 50 mL of the microbial inoculum described above and two were maintained in axenic conditions. Controls (duplicates) composed of WC-Si plus, without A. granulata cells, were inoculated with the microbial community and served as a reference. The flasks were gently aerated with humid filtered air, which generated bubbles with a diameter of around 4mm (a train of 100 bubbles per minute). Aeration was precisely adjusted to ensure identical encounter and shear rates among the treatments. The experiment lasted approximately 40 days and bacteria and A. granulata growth (cell mL^{-1}), aggregates quantification (agreg. mL^{-1}), EPS monosaccharides composition were monitored. Samples were collected at approximately 72-hour intervals in order to monitor the bacterial and phytoplanktonic growth. A 10 mL fraction was fixed with lugol to determine the A. granulata growth and the aggregate concentration by way of

direct counts on a microscope using a Palmer-Malloney 0.1mL chamber. The aggregates were stained with Alcian-Blue (Polyscience, 8GX C.I. 74240) before the counts. All blue-stained particles, with more than 20 mm and with A. granulata cells or frustules, were considered aggregates. Another 10 mL fraction was fixed with 5 % formol to determine the bacterial growth by direct counts on an epifluorescence light microscope with a UV and light source (Zeiss Axioplan 2, Jena, Germany). The aggregates were first mechanically disrupted (Ultra-Turrax T8, Ika, with a S8N-5G shaft tube, Staufen, Germany) and the bacterial cells were then stained using 4'6'-diamidino-2-fenilindol (DAPI) (Porter & Feig, 1980).

After 40 days of growth, A. granulata axenic and microbial co-inoculated cultures were filtered by tangential flow through a hollow fiber cartridge Xampler™with 0.65 mm pore in a QuixStand™Benchtop System, both from A/G Technology (Needham, MA USA). The filtrates were concentrated in a rotatory evaporator at 40°C and dialyzed against distilled water in dialysis tubes with a 12000-14000 D molecular weight cut off. The dialyzed material was freeze-dried, stored at -4° C under gas nitrogen and analyzed by gas chromatography as described below. The monosaccharide composition of the EPS was determined by gas chromatography of the trimethylsilylated derivatives of the methyl-glycosides obtained by methanolysis using 4 M HCl in methanol at 80° C for 24 hours (Barsett et al., 1992; Reinhold, 1972). Mannitol was used as an internal standard. Protein content was determined by the Lowry method (Lowry et al., 1951) and carbohydrate content was determined by phenol-sulfuric assay (Dubois et al., 1956).

Results

The growth of A. granulata was positively influenced by the concomitant bacterial growth, as shown in Fig. 1A. After the sixth day, the microbial co-inoculated culture of A. granulata showed a higher cell density when compared to the axenic culture, confirmed by the "t" test, with "p" values varying between 0.002 and 0.0336. As the cultures were kept under the same light, nutrient and temperature conditions and the dilution effect caused by the microbial inoculum (less than 2%) can be ignored, the only difference between the cultures was the microbial activity.

The growth of the bacterial community was significant, increasing around three orders of magnitude, as shown in Fig. 1B. The bacterial growth was also greater in the A. granulata cultures than in the controls. No bacterial growth was detected in the A. granulata cultures under axenic condition. The behavior of the bacterial community growth seems to indicate a succession pattern of two population groups. The first group reached maximum growth after the 6th day of the experiment, when a second group apparently started to develop, reaching maximum growth the 34th day of the experiment.

We found natural aggregate formation in both axenic and microbial co-inoculated cultures (Fig. 1C), although the pattern and the magnitude of formation were significantly different. In the axenic condition, the aggregates were only formed when the cultures reached the stationary growth phase and in a low concentration. In the microbial co-inoculated culture, on the other hand, the aggregate formation was parallel to the A. granulata growth and was also several orders of magnitude higher than the aggregate formation in the axenic cultures. No type of aggregate formation was observed in the controls.

The monosaccharide composition of the A. granulata EPS from the controls, besides axenic and microbial co-inoculated culture, is shown in Tab.1. A significant increase in the rhamnose proportion in the EPS from the microbial co-inoculated culture and a consequent decrease of the other components, except the N-acetyl-galactosamine, which also increased in the microbial co-inoculated culture. The rhamnose proportion rises from 9.65 % to 14.35 %, which represents an increase of 53 %, while N-acetyl-galactosamine rises from traces to 7.6 %, what means that this monosaccharide could be produced by bacteria. The monosaccharide composition found in the controls differed significantly from the axenic and microbial co-inoculated cultures, and the rhamnose proportion was only 6.2 %. The total amount of EPS isolated from controls and cultures was very low and also significantly different among the treatments (ANOVA $p<0.0001$, Axenic ›Microbial co-inoculated›controls); moreover,

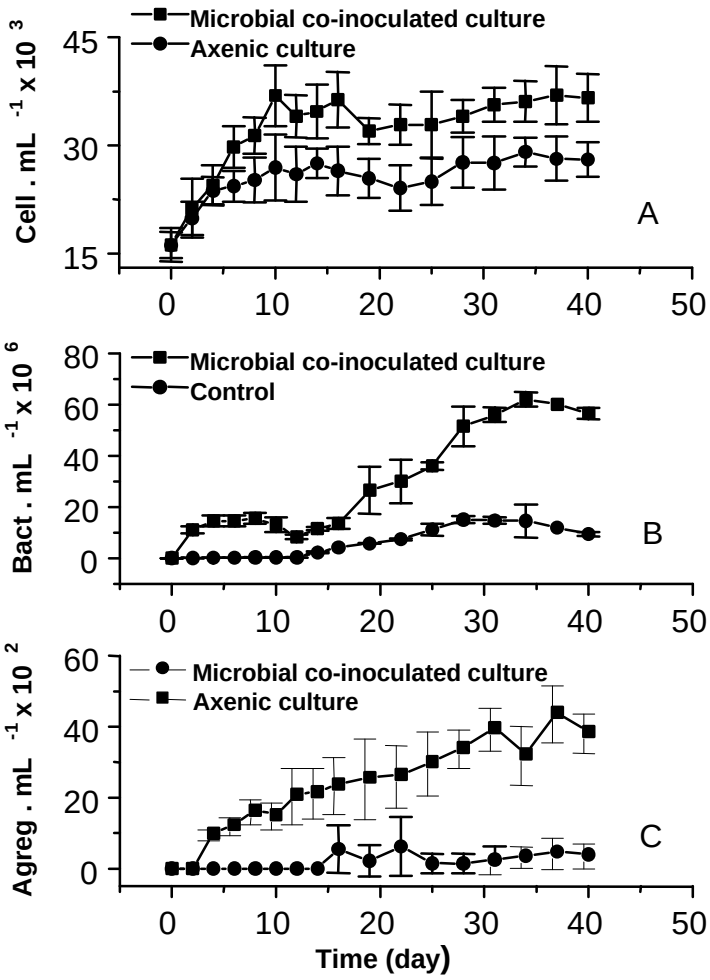

Figure 1: (A) Axenic and microbial co-inoculated culture A. granulata growth during 40 days. (B) Bacterial growth in the microbial co-inoculated cultures and the controls. (C) Aggregate formation in the microbial co-inoculated cultures compared to the axenic cultures of A. granulata. The error bars are the standard deviation (n=4).

Table I: Monomeric composition (percentage) of the EPS produced by the axenic and microbial co-inoculated cultures of A. granulata after 40 days. Total 1 is the sum of the monosaccharide percentages and Total 2 is the dry weight (mg) of the EPS carbohydrate content (phenol-sulfuric assay) found after 40 days in each culture. The interval means the standard deviation (n=2).

MONOSACCHARIDES	AXENIC	CO-INOCULATED	CONTROL
Rhamnose	9.65 ±1.2	14.85 ±0.35	6.2± 4.3
Fucose	11.6 ±0.28	10.35 ±0.77	7.55± 0.35
Xylose	13.25 ±0.63	9.1 ±0.7	6.1± 3.5
Mannose	9.95 ±0.49	7.55 ±0.07	4.7± 2.8
Galactose	7.8 ±1.5	7.65 ±0.63	25.55± 12.5
Galacturonic acid	13.05 ±0.35	10.55 ±1.7	0
Glucoronic acid	12.5 ±0.42	11.7 ±0.14	4.2± 0.7
Glucose	11.5 ±1.8	9.5 ±0.99	45.7± 1.7
N-acetyl-galactosamine	Traces	7.6 ±0.42	0
N-acetyl-glucosamine	10.7 ±1.5	11.15 ±0.77	0
Total 1	100 %	100 %	100 %
Total 2	1.30 ±0.14	1.07 ±0.07	0.20 ±0.01

the EPS production by A. granulata cultures was around 5 times higher than controls. Proteins of the EPS were also attacked by the microbial community, since the protein proportion in the dissolved EPS from axenic cultures was around 5.5 %, while in the EPS from microbial co-inoculated cultures, it decreased to 0.7 %. As a consequence, carbohydrate proportion was higher in the microbial co-inoculated cultures (85%) than in axenic cultures (81%), although the absolute EPS amount, considering only the carbohydrate content, had been higher in the axenic culture.

Discussion

The observation of organic aggregates in natural waters has been extensively described in the last three decades, mainly in marine environments (Alldredge & Silver, 1988; Alldredge et al., 1993; Passow et al., 1994; Passow, 2002;) as has aggregate formation in coagulators and rolling tanks (Shanks & Edmondson, 1989; Kjørboe & Hansen, 1993, Agis et al., 1997). Aggregates formation by freshwater phytoplankters only started to be studied around ten years ago (Grossart & Simon, 1993,1998; Grossart et al., 1997; Brassard & Fish, 2000; Simon et al., 2002; Grossart et al., 2005). However, the spontaneous natural formation of organic aggregates in phytoplanktonic batch cultures had not yet been described. Our results showed significant formation of organic aggregates, with no intervention besides the aeration of the cultures with humid air which is required to make them homogenous and, consequently, to standardize their growth. Passow (2002) demonstrated that bubbles alone are not sufficient to promote TEP formation in bacterial batch cultures. TEP are considered the most important aggregate precursors and its formation on cultures is probably a result of interactions between both bacteria and phytoplankton, not each of them alone (Passow 2002). Aggregation is also controlled by the shear and particle encounter rates (Logan et al., 1995; Engel, 2000; Brassard & Fish, 2000) and, in spite of our experiment had not been designed to monitor such parameters, the bubbling was strictly monitored to ensure that all treatments were identical. Although the particle size and volume were not measured in this work, it is possible to ensure that aggregate formation was greatly increased by concomitant bacterial growth, and both diatom and bacterial cells must be present to start the massive formation of aggregates, since no aggregation was observed in the controls. Moreover, a significant increase in the A. granulata biomass was observed in the microbial co-inoculated cultures, in addition to an increase in the rhamnose proportion of the EPS. Grossart (1999) and Grossart et al. (2005) found similar results for some species; however, some of the diatom strains tested showed growth inhibition when cultivated with bacteria.

Rhamnose accumulation during microbial degradation was found for a freshwater Thalassiosira duostra (Giroldo et al., 2003), and a correlation between the increase of deoxy sugars proportions (fucose and rhamnose) and aggregate formation was found. Hydrophobic properties of these monosaccharides were thought to increase the aggregation capacity of the EPS, but the aggregate concentration was not measured. The refractory characteristic of rhamnose was also mentioned by Aluwihare & Repeta (1999) for Thalassiosira sp, a marine diatom, however no information was found on the relation between this feature and the aggregate concentration increase. Rhamnose and N-acetylhexoamines proportion increase found in our results could be related to EPS production by bacteria, since such monosaccharides are also common constituents of bacterial EPS. However, rhamnose accounted of only 6% of controls EPS and no aminosugars were found. Moreover, the total absolute EPS production was higher in the axenic cultures when compared to non-axenic and controls. On the other hand, the bacterial growth profile was significantly different in the cultures and controls. Recent results found by Grossart et al. (2005) have demonstrated remarkable differences in the bacterial community developed in two diatom species, so it was expected that the bacterial community in the controls was probably different from that in the cultures. However, independently of the rhamnose origin, its proportional increase cannot be ignored, as well as the increase in the hydrophobic properties of the extracellular medium.

Aggregation was notably intense in the microbial co-inoculated cultures, so much so the increase in the rhamnose proportion

was significant, but it could be not the only feature responsible for such a massive aggregation. So extensive is the complexity of such subject that we could enumerated, at least, four more reasons that should be focused on future studies in order to completely understand the results found in this paper: (1) synergistic mutual adsorption of bacteria and A. granulata EPS; (2) the effect of cell density increase when both populations are cultivated together instead of alone; (3) the potential for bacterial lectins recognizing A. granulata EPS and (4) bacterial stimulation for EPS production increase by A. granulata. Besides, other physical-chemical alterations could happen when microbial community and A. granulata cells are cultivated together and future studies involving the dynamics and identification of bacterial populations, as well as chemical analysis on extracellular media, should be carried out in order to identify what factors are also controlling aggregate formation.

AckInowledgements

We would like to thank FAPESP (Processes 99/07766-0 and 03/00084-8) and CNPq (Process 352261/96-0) for financial support.

References

Agis, M., Unanue M., Iriberri, J. & Herndl, G.J. 1997. Bacterial colonization and ectoenzymatic activity in phytoplankton-derived model particles. Part II. Cleavage and uptake of carbohydrates. Microb. Ecol., 36:66-74.

Alcoverro, T., Conte, E. & Mazzella, L. 2000. Production of mucilage by the Adriatic epipelic diatom Cylindrotheca closterium (Bacillariophyceae) under nutrient limitation. J. Phycol., 36:1087-1095.

Alldredge, A.L. & Silver, M.W. 1988. Characteristics, dynamics and significance of marine snow. Limnol. Oceanogr., 20:41-82.

Alldredge, A.L., Passow, U. & Logan, B. 1993. The abundance and significance of a class of large transparent organic particles in the ocean. Deep-Sea Res. Part 1. Oceanogr. Res. Pap., 40:1131-1140.

Aluwihare, L.I. & Repeta, D.J. 1999. A comparison of the chemical characteristics of oceanic DOM and extracellular DOM produced by marine algae. Mar. Ecol. Prog. Ser., 186:105-117.

Barsett, H., Paulsen, B.S. & Habte, Y. 1992. Further characterization of polysaccharides in seeds from Ulmus glabra Huds. Carbohydr. Polym., 18:125-130.

Brassard, P & Fish, S. 2000. The effect of divalent metals and laminar shear on the formation of large freshwater aggregates. Hydrobiologia, 438:143-155.

Dubois, M., Guilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28:350-356.

Engel, A. 2000. The role of transparent exopolymer particles (TEP) in the increase in apparent particle stickiness (a) during the decline of a diatom bloom. J. Plankton Res., 22:485-497.

Giroldo, D., Paulsen, B.S. & Vieira, A.A.H. 2003. The relative increase of deoxy sugars during microbial degradation of an extracellular polysaccharide released by a tropical freshwater Thalassiosira sp. (Bacillariophyceae). J. Phycol., 39:1109-1111.

Grossart, H.P. 1999. Interactions between marine bacteria and axenic diatoms (Cylindrotheca fusiformis, Nitzschia laevis, and Thalassiosira weissflogii) incubated under various conditions in the lab. Aquat. Microb. Ecol., 19:1-11.

Grossart, H.P. & Simon, M. 1993. Limnetic macroscopic organic aggregates (lake snow): Occurrence, characteristics, and microbial dynamics in Lake Constance. Limnol. Oceanogr., 38:532-546.

Grossart, H.P. & Simon, M.1998. Significance of limnetic organic aggregates (lake snow) for the sinking flux of particulate organic matter in a large lake. Aquat. Microb. Ecol., 15:115-125.

Grossart, H.P., Simon, M. & Logan, B.E. 1997. Formation of macroscopic aggregates (lake snow) in a large lake: the significance of transparent exopolymer particles, phytoplankton, and zooplankton. Limnol. Oceanogr., 42:1651-1659.

Grossart, H.P., Levold, F., Allgaier, M., Simon, M. & Brinkhoff, T. 2005. Marine diatoms species harbour distinct bacterial communities. Environ. Microbiol., 7:860-873.

Guillard, R.R.L. & Lorenzen, C.J. 1972. Yellow-green algae with chlorophyllide-c. J. Phycol., 8:10-14.

Kiørboe, T. & Hansen, J.L.S. 1993. Phytoplankton aggregate formation: observation of patterns and mechanisms of cell sticking and the significance of exoploymer material. J. Plankton Res., 15:993-1018.

Leppard, G.G. 1995. The characterization of algal and microbial mucilages and their aggregates in aquatic ecosystems. Sci.Total Environ., 1-3:103-131.

Logan, B.E., Passow, U., Alldredge, A.L., Grossart, H.P. & Simon, M. 1995. Rapid formation and sedimentation of large aggregates is predictable from coagulation rates (half-lives) of transparent exopolymer particles (TEP). Deep-Sea Res. Part 2, Top. Stud. Oceanogr., 42:230-214.

Lowry, O.H., Rosebrough, N.H., Farr, A.L. & Randal, R.J. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem., 193:265-275.

Passow, U., Alldredge, A.L. & Logan, B.E. 1994. The role of particulate carbohydrate exudates in the flocculation of diatom blooms. Deep-Sea Res., 41:335-357.

Passow, U. 2002. Production of transparent exopolymer particles (TEP) by phyto- and bacterioplankton. Mar. Ecol. Prog. Ser., 236:1-12.

Porter, K.G. & Feig, Y. 1980. The use of DAPI for identification and enumeration of bacteria and blue green algae. Limnol. Oceanogr., 25:943-948.

Reinhold, V.N. 1972. Gas-liquid chromatographic analysis of constituent carbohydrates in glycoproteins. Methods Enzymol., 25:244-249.

Shanks, A.L. & Edmondson, E.W. 1989. Laboratory-made artificial marine snow: a biological model of the real thing. Mar. Biol., 101:463-470.

Simon, M., Grossart, H., Schweitzer, B. & Ploug, H. 2002. Microbial ecology of organic aggregates in aquatic ecosystems. Aquat. Microb. Ecol., 28:175-211.

Stoderegger, K. & Herndl, G. 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol. Oceanogr., 43:877-884.

Vieira, A.A.H. 1983. Purification of phytoplankton cultures with Dakin solution. Rev. Microbiol., 14:202-203.

Received: 10 August 2005
Accepted: 11 April 2006