Vol. 11: 243–248, 2011
doi: 10.3354/ab00309

AQUATIC BIOLOGY
Aquat Biol

Published online January 25

Flocculating activities of polysaccharides released from the marine mat-forming cyanobacteria *Microcoleus* and *Lyngbya*

Li Chen[1,2], Tianli Li[1], Liang Guan[1], Yawei Zhou[1], Pengfu Li[1,*]

[1]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, PR China

[2]Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, PR China

ABSTRACT: Mat-forming cyanobacteria of the genera *Microcoleus* and *Lyngbya* were isolated from the Tainan Saltworks, China. The released polysaccharides (RPSs) from the 2 cyanobacterial isolates flocculated kaolin clay from suspensions, and this flocculating activity decreased with increasing pH from 3 to 10.5. The flocculating activities increased with increasing metal ion concentrations and reached plateaus when the concentrations of monovalent cations (Na^+ or K^+) and divalent cations (Mg^{2+} or Ca^{2+}) increased to 0.4 and 0.2 mol l^{-1}, respectively. Compared with the previously reported data about the flocculating activities of exopolysaccharide from the freshwater cyanobacterium *Phormidium* J-1, the presence of higher concentrations of metal ions was required for the flocculating activities of the RPSs from the 2 marine cyanobacteria studied here. The production of extracellular bioflocculants by the benthic cyanobacteria could be of considerable importance in the flocculation and sedimentation of clay particles in brines, which in turn would allow light to penetrate to the sediment–water interface.

KEY WORDS: Flocculating activity · Released polysaccharide · Marine cyanobacteria · *Microcoleus* sp. · *Lyngbya* sp.

INTRODUCTION

Some freshwater cyanobacteria, such as benthic cyanobacteria *Phormidium* sp. strain J-1, *Anabaenopsis circularis* PCC6720 and *Calothrix desertica* PCC7102 and planktonic cyanobacteria *Anabaena* sp. N1444 and *Anabaena* sp. PC-1, have been reported to produce exopolysaccharides with flocculating activity (Fattom & Shilo 1984, Bar-Or & Shilo 1987, 1988, Bender et al. 1994, Choi et al. 1998). However, up to now, no marine cyanobacteria have been reported to produce exopolysaccharides with flocculating activity.

The production of extracellular bioflocculants by benthic cyanobacteria is of considerable ecological importance. Aggregation and flocculation of suspended particles by bioflocculants might have a critical role in allowing light to penetrate to the sediment–water interface, thus facilitating the survival and growth of the benthic cyanobacteria that occupy a low-light zone (Fattom & Shilo 1984, Bender et al. 1994). Furthermore, through sedimentation of particles carrying absorbed nutrients, the flocculant might enhance the nutrient accumulation at the water–sediment interface where they would be more easily available to the benthic cyanobacteria (Fattom & Shilo 1984). Additionally, it is proposed that attachment of cyanobacteria to the benthos can be facilitated by the mutual flocculation of cyanobacteria and clay particles, which is promoted by the extracellular bioflocculant of cyanobacteria (Bar-Or & Shilo 1988, Sutherland 2001).

Cyanobacterial mats commonly occur in coastal and hypersaline environments (Ladakis et al. 2006, Abed et al. 2008, Kremer et al. 2008). Coastal cyanobacterial mats play an important role in structuring and biostabilizing coastal sediments and influencing coastal morphodynamics (Yallop et al. 1994, Stal 2003, Kremer et al.

*Email: pengfuli@nju.edu.cn

2008). Cyanobacterial mats in salterns are considered good for salt production through the prevention of brine permeation (Javor 2002, Liu et al. 2002). *Microcoleus* sp. and *Lyngbya* sp. are the predominating species of cyanobacterial mats in saltworks (Hua & Liu 1993). In the present study, these 2 cyanobacteria were to release polysaccharides (RPSs) during growth, and we investigated the flocculating properties of RPS from *Microcoleus* sp. (M-RPS) and from *Lyngbya* sp. (L-RPS).

MATERIALS AND METHODS

Microorganisms and chemicals. *Microcoleus* sp. and *Lyngbya* sp. were isolated from the Tainan Saltworks, Jiangsu Province, PR China. Morphological classification of cyanobacteria was done according to Hua & Liu (1993). The 16S rRNA gene sequences of *Microcoleus* sp. and *Lyngbya* sp. were deposited in GenBank, and their accession numbers are HQ343413 and HQ343414, respectively. The 2 unialgal isolates were grown at 25 ± 2°C in ASN-III medium (Rippka et al. 1979). The cultures were continuously aerated by gently bubbling them with filtered air. A light intensity of 20 µmol photons m^{-2} s^{-1} was provided by white fluorescent tubes under a 12 h light:12 h dark cycle.

Kaolin (Sigma) clay slurries were prepared by dispersing clay particles (particle size: 0.1 to 4 µm) in 20 mmol l^{-1} Tris buffer. NaCl, KCl, $MgCl_2$ and $CaCl_2$ were purchased from Amesco, and all other chemicals used were analytical grade.

Preparation of RPS stock solutions. Cyanobacterial cells of exponential growth phase were removed by centrifugation (6000 × *g*, 20 min). The supernatant containing RPS was filtered first through a 0.45 µm and then a 0.22 µm mixed cellulose ester membrane to remove possible remaining cell debris. The filtered supernatant was transferred into dialysis tubes with a cutoff molecular weight of 7000 Da, dialyzed against distilled water at 4°C for 72 h and further concentrated by a rotary evaporator under reduced pressure at 45°C. Since lyophilized RPSs can not dissolve completely in distilled water and Tris buffer solution, the concentrated RPS solutions were used for preparing stock solutions. Carbohydrate content of RPS solutions were measured by the phenol–sulfuric acid method (Dubois et al. 1956) using glucose as a reference. The actual polysaccharide content in the RPS solution was calculated by multiplying the measured carbohydrate content (expressed as glucose equivalent) with experimentally determined correction factors of 7.01 and 3.53 for M-RPS and L-RPS, respectively. After mixing with 1 mol l^{-1} Tris buffer, the concentrated RPS solutions with final Tris concentrations of 20 mmol l^{-1} were used as stock solutions.

Chemical characterization of RPSs. The protein content was determined by the method of Bradford (1976) using bovine serum albumin as a reference. The uronic acid content was assayed using the carbazole method (Galambos 1967) and expressed as glucuronic acid equivalents. Quantitative analysis of sulfate was performed by the method of barium chloride–gelatin (Dodgson & Price 1962). The fatty acid ester content of RPS was estimated after hydrolysis with 0.5 mol l^{-1} KOH (50°C, 90 min), extraction with petroleum ether, evaporation of the solvent and weighing (Bar-Or & Shilo 1987). Lyophilized RPSs were hydrolyzed with 2 mol l^{-1} trifluoroacetic acid at 120°C for 3 h. For qualitative analysis, sugars in the hydrolysate were derivatized with 1-phenyl-3-methyl-5-pyrazolone for HPLC analysis (Strydom 1994). HPLC runs were performed on a Zorbax SB-C18 column (150 mm high × 4.6 mm inside diameter) and a UV detector (245 nm) with a mobile phase of 0.1 mol l^{-1} phosphate buffer–acetonitrile (83:17, v/v). For qualitative and quantitative analysis, neutral sugars in the hydrolysate were converted into acetylated aldononitrile derivatives (Li et al. 1982). After the dried hydrolysate was dissolved in 0.5 ml pyridine, 7 mg hydroxylamine hydrochloride was added. The mixture was reacted at 90°C for 30 min and cooled to room temperature. After the addition of 0.5 ml acetic anhydride, the reaction was continued at 90°C for a further 30 min. The acetylated aldononitrile derivatives were analyzed by gas–liquid chromatography on a HP6890 instrument equipped with a HP 55% phenyl methyl siloxane capillary column (30 m × 0.25 mm × 0.25 µm) and a flame ionization detector. The temperature profile was programmed as follows: 146°C for 2 min, from 146 to 210°C at 2°C min^{-1}, and from 210 to 280°C at 30°C min^{-1}. The carrier gas was N_2 and the flow rate was 1.2 ml min^{-1}.

Determination of flocculating activity. Kaolin clay slurry was added into 7 ml test tubes containing RPS stock solution, and then the metal ion solution in 20 mmol l^{-1} Tris buffer was added. The final volume was adjusted to 5 ml with 20 mmol l^{-1} Tris buffer, and the final kaolin clay concentration was 3.5 g l^{-1}. The test tubes were vortexed thoroughly for 30 s and allowed to stand at room temperature (20°C) for 5 min. The turbidity of the upper 1.5 ml was measured at 550 nm (optical density; OD_{550}). A control without RPS was assayed in the same manner. Three replicates were carried out for each test. The flocculating activity, expressed as the decrease of OD_{550} relative to the reference, was calculated according to the following equation (Buelna et al. 1990):

$$\text{Flocculating activity (\%)} = (1 \times A/B) \times 100,$$

where A is OD_{550} of the sample, and B is OD_{550} of the reference in the corresponding control tube (without RPS).

To investigate the RPS concentration on the flocculating activity, various volumes of RPS stock solutions were added into test tubes. To investigate the effect of metal ions on the flocculating activity, various concentrations of metal ions including NaCl, KCl, $MgCl_2$ or $CaCl_2$ were added to the test tubes. To examine the effect of pH on flocculating activity, the Tris buffer was adjusted with HCl in the pH range of 3.0 to 10.5.

RESULTS AND DISCUSSION

Chemical characterization of M-RPS and L-RPS

No proteins were detected in M-RPS and L-RPS solutions. The monosaccharide composition of M-RPS and L-RPS is reported in Table 1. HPLC analysis of the M-RPS and L-RPS hydrolysates showed the presence of galacturonic acid and glucuronic acid. The sulfate contents of M-RPS and L-RPS were 23.5 and 16.0%, respectively. The fatty acid ester contents of M-RPS and L-RPS were 4.2 and 7.2%, respectively.

Table 1. *Microcoleus* sp. and *Lyngbya* sp. Monosaccharide composition of the released polysaccharides from *Microcoleus* sp. (M-RPS) and *Lyngbya* sp. (L-RPS). nd: no data

Monosaccharide	M-RPS	L-RPS
Neutral sugars[a]		
Rhamnose	1.4	4.8
Fucose	1.2	4.3
Xylose	1.3	nd
Mannose	1.4	1.0
Glucose	1.0	2.4
Galactose	1.2	3.4
Acidic sugars		
Uronic acid[b]	4.1	7.6

[a]expressed as molar ratio
[b]expressed as % of polysaccharide dry weight

Effects of M-RPS and L-RPS concentration on flocculating activity

In the Tainan Saltworks, the cyanobacterial mat was composed mainly of *Microcoleus*, *Lyngbya*, *Oscillatoria* and *Phormidium* (Hua & Liu 1993). *Microcoleus* sp., *Lyngbya* sp. and *Oscillatoria* sp. were isolated from the saltworks. Our preliminary experiments showed that the RPSs from all 3 cyanobacteria showed flocculating activity, and that high concentrations of metal ions were required for the flocculating activity. Flocculating activities of the RPSs from *Microcoleus* sp. and *Lyngbya* sp. were investigated further. The flocculating activity of M-RPS increased following the increase of M-RPS concentration from 0 to 40 mg l^{-1} and decreased thereafter (Fig. 1a). As for L-RPS, the peak flocculating activity occurred at 20 mg l^{-1} L-RPS (Fig. 1b). This phenomenon, with respect to excess flocculant, has been reported by others (Fattom & Shilo 1984, Prasertsan et al. 2006, Zheng et al. 2008).

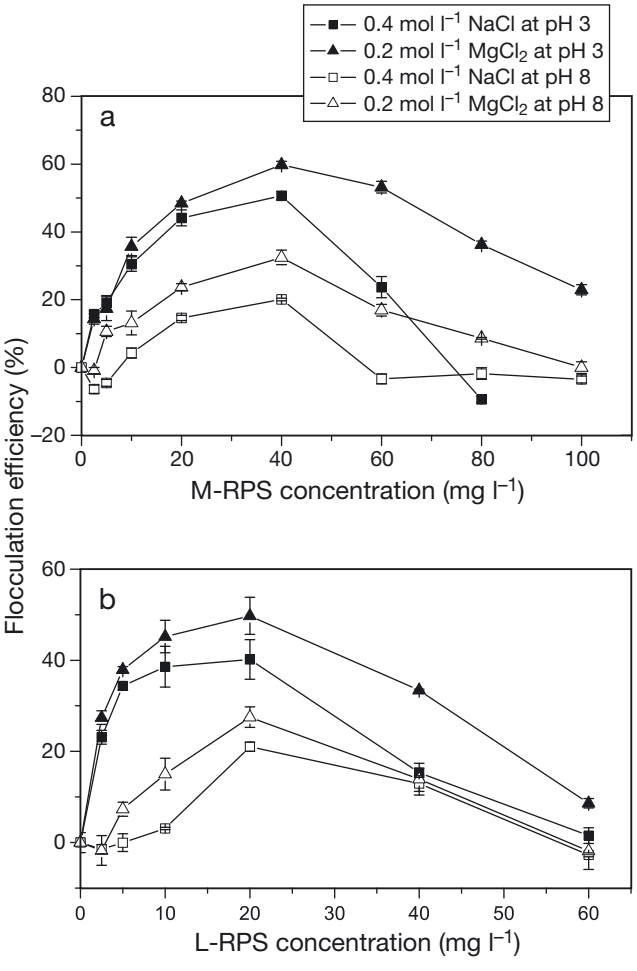

Fig. 1. *Microcoleus* sp. and *Lyngbya* sp. Effects of the concentration of released polysaccharide from (a) *Microcoleus* sp. (M-RPS) and (b) *Lyngbya* sp. (L-RPS) on flocculating activity in metal ion aqueous solution using 3.5 g l^{-1} kaolin at pH 3 and pH 8. Each point with associated error bars represents mean ± SD (n = 3)

Effects of pH and various metal ions on flocculating activity

The flocculating activities of both M-RPS and L-RPS decreased with the increasing pH (Fig. 2). This suggested that the hydroxide ion (OH^-) may interfere with the complex formation of RPSs and kaolin clay mediated by metal ions, leading to the suspension of kaolin clay (Prasertsan et al. 2006). This is consistent with the

flocculating activities of the polysaccharide bioflocculants produced by *Anabaena* sp. PC-1 and the bacterium *Bacillus* sp. F19 (Choi et al. 1998, Zheng et al. 2008). The effects of various metal ions on flocculating activity of M-RPS and L-RPS were examined using NaCl, KCl, $MgCl_2$ and $CaCl_2$ (Figs. 3 & 4). The presence of metal ions was essential for the flocculating activity. At pH 3 or pH 8, the flocculating activity of both M-RPS and L-RPS increased with increasing metal ion concentrations and reached their plateaus when the concentrations of NaCl, KCl, $MgCl_2$ and $CaCl_2$ were increased to 0.4, 0.4, 0.2 and 0.2 mol l^{-1}, respectively. Fattom & Shilo (1984) reported that exopolysaccharide of the freshwater benthic cyanobacterium *Phormidium* J-1 can flocculate bentonite only above critical concentrations of monovalent and divalent metal ions, and 0.8 mmol l^{-1} Mg^{2+} and 12 mmol l^{-1} Na^+ are sufficient to achieve maximum flocculating activity. Our results showed that the presence of higher concentrations of metal ions was required for the flocculating activities of the RPSs from marine cyanobacteria *Microcoleus* sp. and *Lyngbya* sp. compared with the freshwater cyanobacterium *Phormidium* J-1. Fattom & Shilo (1984) suggested that the flocculation process induced by divalent metal ions is based on the bridging mechanism. There is no report on the mechanism that induces flocculation by monovalent metal ions. We speculate that, as with bivalent metal ions, the presence of monovalent metal ions may also decrease the negative electrical charge density of the clay particles and of the bioflocculant molecules and increase the flocculation efficiency.

Solar salterns can be modeled as flow-through pond systems in which seawater flows in from the seawater inlets and passes through the crystallizer ponds. In a typical coastal solar saltern, seawater is pumped into a series of shallow evaporating ponds where the salinity of seawater is concentrated up to saturation of NaCl (Javor 2002). As the brines slowly flow downstream to the crystallizers, the concentrations of the components that include Na^+, K^+, Mg^{2+} and Ca^{2+} are passively increased. In the Guangrao Saltworks, the concentrations of K^+, Mg^{2+} and Ca^{2+} are in the range of 8.1 to 77.1, 125.9 to 861.1 and 10.3 to 20.6 mmol l^{-1}, respectively (Li et al. 2000). In the saltern of the Bretagne, France, the pH values of the brine and sediment are in

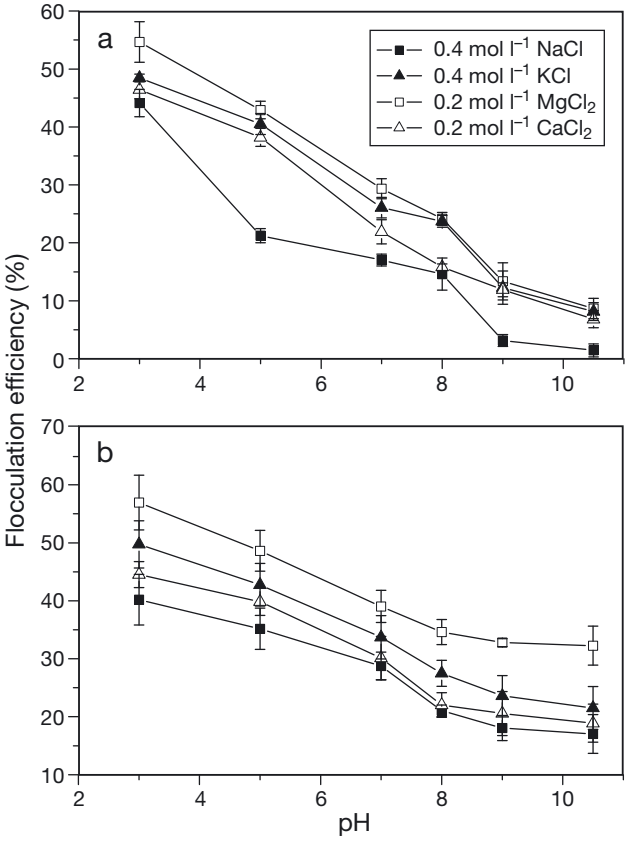

Fig. 2. *Microcoleus* sp. and *Lyngbya* sp. Effects of pH on the flocculating activity of released polysaccharide from (a) *Microcoleus* sp. (M-RPS) and (b) *Lyngbya* sp. (L-RPS) using 3.5 g l^{-1} kaolin and 20 mg l^{-1} M-RPS and 20 mg l^{-1} L-RPS. Each point with associated error bars represents mean ± SD (n = 3)

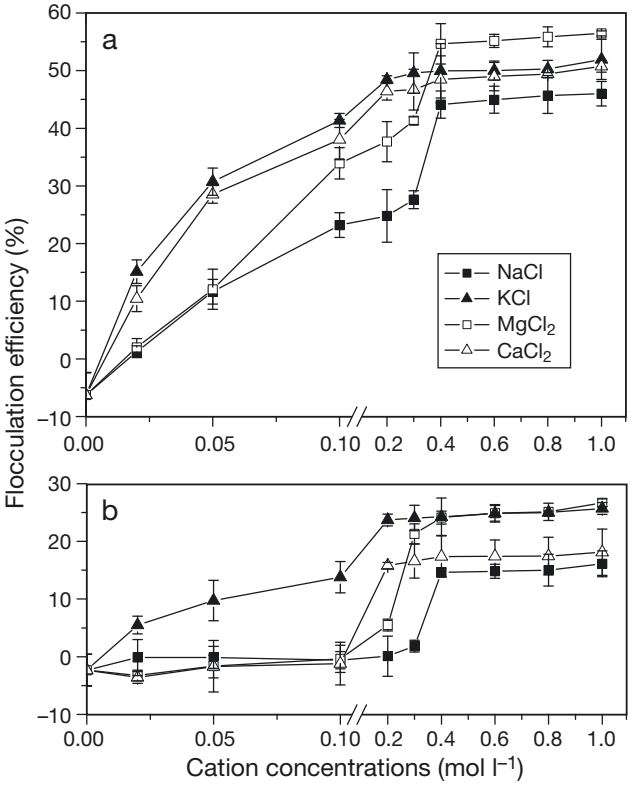

Fig. 3. *Microcoleus* sp. Effects of cation concentrations on the flocculating activity of released polysaccharide from *Microcoleus* sp. (M-RPS) using 3.5 g l^{-1} kaolin and 20 mg l^{-1} M-RPS at (a) pH 3 and (b) pH 8. Each point with associated error bars represents mean ± SD (n = 3)

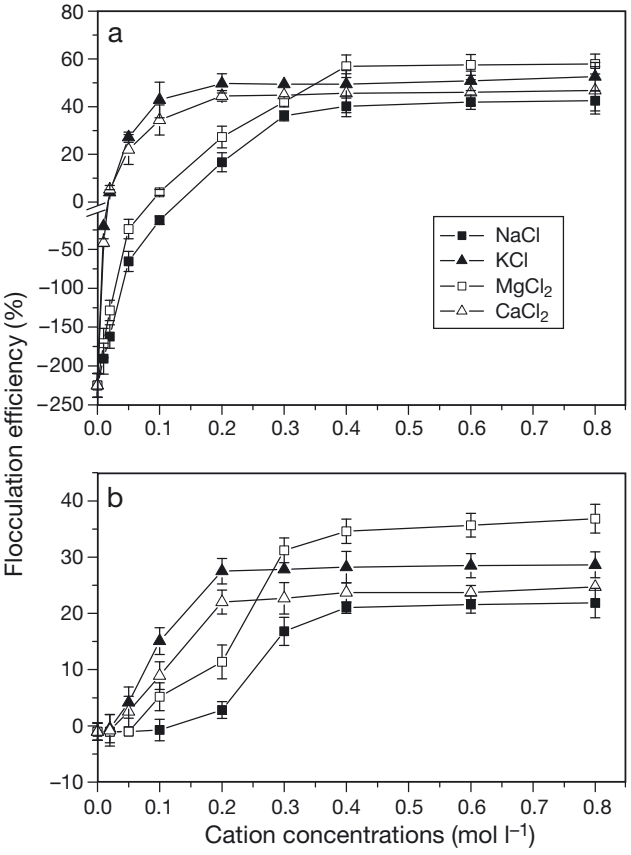

Fig. 4. *Lyngbya* sp. Effects of cation concentrations on the flocculating activity of released polysaccharide from *Lyngbya* sp. (L-RPS) using 3.5 g l^{-1} kaolin and 20 mg l^{-1} L-RPS at (a) pH 3 and (b) pH 8. Each point with associated error bars represents mean ± SD (n = 3)

the range of 7.1 to 9.5 and 6.2 to 6.9, respectively (Giani et al. 1989). Our data indicate that M-RPS and L-RPS might maintain the flocculating activity in the saltern brine. The production of extracellular bioflocculant by the benthic cyanobacteria could be of considerable significance in the flocculation and sedimentation of clay particles in the brines, thus allowing light to penetrate to the sediment–water interface. Furthermore, the mutual flocculation of benthic cyanobacteria and clay particles is promoted by the production of extracellular flocculants, thus facilitating attachment to the benthos (Bar-Or & Shilo 1988). Therefore, the production of extracellular bioflocculant by *Microcoleus* sp. and *Lyngbya* sp. might facilitate the survival and growth of the benthic cyanobacteria in the saltern. By contrast, in our previous report, *Aphanothece halophytica*, a cyanobacterium forming surface bloom in the saltern, was found to produce RPS with clay-dispersing activity, which inhibits mutual flocculation of the cyanobacterium and clay particles and may prevent the sedimentation of the cyanobacterium in the brine (Chen et al. 2010). Interestingly, RPS from *A. halophytica* with clay-dispersing activity might facilitate its planktonic growth, and RPSs from *Microcoleus* sp. and *Lyngbya* sp. with flocculating activity might facilitate their benthic growth. Compared with other cyanobacteria, chemical compositions of M-RPS, L-RPS and RPS from *A. halophytica* have no distinctive characterization (De Philippis & Vincenzini 1998, Li et al. 2001a,b, Pereira et al. 2009). The relationship between the different activities of M-RPS and L-RPS and RPS from *A. halophytica* and their polysaccharide compositions is not clear and needs further investigation.

CONCLUSIONS

Compared with the previously reported data about the flocculating activities of the exopolysaccharide from the freshwater cyanobacterium *Phormidium* J-1, the presence of higher concentrations of metal ions was required for the flocculating activities of the RPSs from the marine benthic cyanobacteria *Microcoleus* sp. and *Lyngbya* sp. The production of extracellular bioflocculant by the benthic cyanobacteria could be of importance for flocculation and sedimentation of clay particles in brines, which in turn would allow light to penetrate to the sediment–water interface.

Acknowledgements. The authors are grateful to 2 referees for their valuable comments. This study was sponsored by the National Natural Science Foundation of China (No. 30670400) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

LITERATURE CITED

Abed RMM, Kohls K, Schoon R, Scherf AK and others (2008) Lipid biomarkers, pigments and cyanobacterial diversity of microbial mats across intertidal flats of the arid coast of the Arabian Gulf (Abu Dhabi, UAE). FEMS Microbiol Ecol 65:449–462

Bar-Or Y, Shilo M (1987) Characterization of macromolecular flocculants produced by *Phormidium* sp. strain J-1 and by *Anabaenopsis circularis* PCC6720. Appl Environ Microbiol 53:2226–2230

Bar-Or Y, Shilo M (1988) The role of cell-bound flocculants in coflocculation of benthic cyanobacteria with clay particles. FEMS Microbiol Ecol 53:169–174

Bender J, Rodriguezeaton S, Ekanemesang UM, Phillips P (1994) Characterization of metal-binding bioflocculants produced by the cyanobacterial component of mixed microbial mats. Appl Environ Microbiol 60:2311–2315

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Buelna G, Bhattarai KK, de la Noüe J, Taiganides EP (1990) Evaluation of various flocculants for the recovery of algal biomass growth on pig-waste. Biol Wastes 31:211–222

Chen L, Men X, Ma MR, Li PF and others (2010) Polysaccharide release by *Aphanothece halophytica* inhibits cyanobacteria/clay flocculation. J Phycol 46:417–423

Choi CW, Yoo SA, Oh IH, Park SH (1998) Characterization of an extracellular flocculating substance produced by a planktonic cyanobacterium, *Anabaena* sp. Biotechnol Lett 20:643–646

De Philippis R, Vincenzini M (1998) Exocellular polysaccharide from cyanobacteria and their possible applications. FEMS Microbiol Rev 22:151–175

Dodgson KS, Price RG (1962) A note on the determination of the ester sulphate content of sulphated polysaccharides. Biochem J 84:106–110

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method of determination of sugars and related substances. Anal Chem 28:350–356

Fattom A, Shilo M (1984) *Phormidium* J-1 bioflocculant: production and activity. Arch Microbiol 139:421–426

Galambos JT (1967) The reaction of carbazole with carbohydrates. I. Effect of borate and sulfamate on the carbazole color of sugars. Anal Biochem 19:119–132

Giani D, Seeter J, Giani L, Krumbein WE (1989) Microbial mats and physicochemistry in a saltern in the Bretagne (France) and in a laboratory scale saltern model. FEMS Microbiol Ecol 62:151–162

Hua D, Liu ZL (1993) The algal flora in Tainan saltworks of Jiangsu. J Nanjing Univ Sci Technol (Nat Sci) 29:77–85

Javor BJ (2002) Industrial microbiology of solar salt production. J Indian Microbiol Biotechnol 28:42–47

Kremer B, Kazmierczak J, Stal LJ (2008) Calcium carbonate precipitation in cyanobacterial mats from sandy tidal flats of the North Sea. Geobiology 6:46–56

Ladakis M, Dassenakis M, Pantazidou A (2006) Nitrogen and phosphorus in coastal sediments covered by cyanobacteria mats. J Soils Sediments 6:46–54

Li TL, Wu CX, Zhang YX (1982) Studies of gas chromatographic analysis of saccharides and alditols: some improvements in the analysis of acetylated aldononitriles by gas chromatography. Fenxi Huaxue (Chin J Anal Chem) 10:272–276 (in Chinese with English Abstract)

Li PF, Liu ZL, Ge HT, Shen RJ, Shen H (2000) Effects of salinity and nutritional limitation on the growth and exopolysaccharide production of *Aphanothece halophytica.* J Nanjing Univ Sci Technol (Nat Sci) 36:585–591

Li PF, Harding SE, Liu ZL (2001a) Cyanobacterial exopolysaccharides: their nature and potential biotechnological applications. Biotechnol Genet Eng Rev 18:375–404

Li PF, Liu ZL, Xu R (2001b) Chemical characterisation of the released polysaccharide from the cyanobacterium *Aphanothece halophytica* GR02. J Appl Phycol 13:71–77

Liu ZL, Li PF, Liu XX (2002) Culturing artificial algal mats to improve the salt yield and quality in saltworks. Ecol Eng 18:379–383

Pereira S, Zille A, Micheletti E, Moradas-Ferreira P, De Philippis R, Tamagnini P (2009) Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS Microbiol Rev 33:917–941

Prasertsan P, Dermlim W, Doelle H, Kennedy JF (2006) Screening, characterization and flocculating property of carbohydrate polymer from newly isolated *Enterobacter cloacae* WD7. Carbohydr Polym 66:289–297

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Stal LJ (2003) Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. Geomicrobiol J 20:463–478

Strydom DJ (1994) Chromatographic separation of 1-phenyl-3-methyl-5-pyrazolone-derivatized neutral, acidic and basic aldoses. J Chromatogr A 678:17–23

Sutherland IW (2001) Exopolysaccharides in biofilms, flocs and related structures. Water Sci Technol 43:77–86

Yallop ML, de Winder B, Paterson DM, Stal LJ (1994) Comparative structure, primary production and biogenic stabilization of cohesive and non-cohesive marine sediments inhabited by microphytobenthos. Estuar Coast Shelf Sci 39:565–582

Zheng Y, Ye ZL, Fang XL, Li YH, Cai WM (2008) Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. Bioresour Technol 99:7686–7691

Editorial responsibility: Warwick Vincent, Sante-Foy, Quebec, Canada

Submitted: June 23, 2010; Accepted: November 8, 2010
Proofs received from author(s): January 20, 2011