ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 1, pp. 84–89. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © O.A. Fuchedzhi, S.A. Konnova, A.S. Boiko, V.V. Ignatov, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 1, pp. 96–102.

EXPERIMENTAL ARTICLES

Role of the Clasping-Leaved Pondweed (*Potamogeton perfoliatus*) Polysaccharides in Formation of Its Bacterial Environment

O. A. Fuchedzhi[a], S. A. Konnova[b,1], A. S. Boiko[a], and V. V. Ignatov[a]

[a] *Institute for Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov*
[b] *Chernyshevskii Saratov State University*

Received March 12, 2007

Abstract—The effect of the polysaccharides of clasping-leaved pondweed (*Potamogeton perfoliatus*) on the formation of a bacteriocenosis of this plant was demonstrated by research on chemoreception, relative surface hydrophobicity, and the growth characteristics of the members of five bacterial genera abundant in this micro biocenosis. The plant heteropolysaccharides of anionic and cationic nature were found to participate in selective stimulation or inhibition of growth of some microbial groups in surrounding water. These findings improve our understanding of the spectrum of physiological activity of glycopolymers of diverse origin.

Key words: polysaccharides, microbial cell surface, plant–microbe associations.

DOI: 10.1134/S0026261708010128

Plant–microbe associations of the shallow coastal zone play an important part in the self-purification of freshwater basins [1, 2]. Metabolites of diverse origin, including carbohydrates and amino acids, participate in formation of the relationship between phytoplankton, microorganisms, and higher aquatic vegetation (HAV) [2–4]. Trophic relations are usually the basis of interactions within biocenoses [5]. Macrophytes are believed to play the main role in the regulation of the flows of carbon, soluble mineral compounds, and oxygen in freshwater ecosystems [6–8].

Clasping-leaved pondweed (*Potamogeton perfoliatus*) is among the most widespread freshwater macrophytes of the Volga reservoirs; it is an indicator of eutrophic, flowing freshwater reservoirs with high carbonate content, slimy–sandy bottom sediments, and contaminated aquatic environment [9–11]. A number of authors have demonstrated that bacteria of the genera *Pseudomonas, Xanthomonas, Micrococcus*, and *Escherichia* are permanently present in pondweed microbiocenoses [12–14]. Azospirilla can form efficient associations with rice, which is a semisubmerged aquatic plant [15]. The interactions between macrophytes and the microorganisms are mostly associative; however, the mechanisms and metabolites most important for their formation are still poorly studied [15, 16]. The intravital and postlethal supply of carbohydrates and polysaccharides from HAV into the environment can participate in environmental processes and may have diverse effects on both the environment and its inhabitants, including the growth and metabolic activity of accompanying bacteria [17–19]. The role of the major structural and storage polysaccharides (PS) is generally understood; the minor water-soluble PSs are, however, beyond the scope of researchers. Their relatively low content and pronounced diversity suggest their role in communication between the organisms in cenoses.

The goal of the present work was to determine the role of vegetative water-soluble polysaccharides in the formation of a bacterial cenosis, exemplified by clasping-leaved pondweed (*Potamogeton perfoliatus*) and the microorganisms abundant in the tangle of freshwater macrophytes of slowly flowing water.

MATERIALS AND METHODS

Bacterial cultures used in the present work included gram-negative (*Xanthomonas campestris* B-610, *Escherichia coli* K12, *Pseudomonas fluorescens* B-1471, *Azospirillum brasilense* Sp245) and gram-positive organisms (*Micrococcus luteus* B-109). The strains were obtained from the collection of the Institute for Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov. Except for *X. campestris*, which was maintained in glucose–salt medium [20], the organisms were grown in liquid LB medium [20].

Viable clasping-leaved pondweed (*Potamogeton perfoliatus* L.) plants with well-developed biomass were collected in the shallows of Volga near Saratov in July 2004 according to the accepted procedure for collecting aquatic plants [21]. Averaged biomass samples containing several whole plant specimens were used for analysis. The plants were washed with tap water and fixed by freezing at –20°C.

[1] Corresponding author; e-mail: konnova@ibppm.sgu.ru

The polysaccharide fractions were extracted from plant biomass by two methods, water and water–alcohol extraction [22]. The extract was treated with a 10% water solution of lead acetate in order to remove the pigments. The carbohydrate mixture was fractioned by gel filtration on a Sephadex G-50 (Pharmacia, Sweden) column (1.8 × 55 cm). Pyridine–acetate buffer (0.025 M, pH 4.1) was used for elution. The carbohydrate elution profile was determined by means of the reaction with phenol and sulfuric acid [23]. The molecular mass was determined by gel filtration. The columns contained Sepharose CL-4B and Sephadex G-50; the calibration curve was determined using the standard dextrans with molecular masses of 1×10^4 (Loba Chemie, Austria), 2×10^4 (Ferak, Germany), 4×10^4 (Fluka, Switzerland), 7×10^4 (Fluka, Switzerland), 1.1×10^5 (Fluka, Switzerland), 2.3×10^5 (Sigma, United States), and 5×10^5 Da (Pharmacia, Sweden).

The monosaccharide composition was determined by thin-layer and gas–liquid chromatography. The polysaccharides were hydrolyzed with 4 N trifluoroacetic acid in sealed ampoules (100°C, 4 h). Thin-layer chromatography of the hydrolysates was carried out on cellulose-covered plates. The mixture of pyridine, ethyl acetate, water, and acetic acid in the volume ratio of 5 : 5 : 3 : 1 was used as the solvent system. The relevant monosaccharides were revealed by an anisidine phthalate solution in water-saturated butanol. Gas–liquid chromatography was carried out on Chrom-5 (Czech Republic) and Hewlett-Packard 5890 (United States) chromatographs.

Bacterial cultures were centrifuged and resuspended in 0.9% NaCl solution (10^6 cells/ml).

Microbial chemotaxis to the pondweed polysaccharide fractions was determined by means of the so-called Adler test [24]. The concentration of all glycans was 10^{-3} mM. Dextrans of the same molecular mass were used for control. The cells from the late exponential culture (20 μl) were applied to the center of a petri dish with semisolid agar containing the pondweed polysaccharide under investigation. The taxis was determined after 18 h of incubation by the chemotactic ring of motile cells.

The relative hydrophobicity of bacteria was determined by the salt aggregation test [25]. Fresh microbial cultures were resuspended in a phosphate buffer. The turbidity of bacterial suspensions was adjusted to the same level (1.4 U at 420 nm). A Specol 221 photoelectric colorimeter (Carl Zeiss, GDR) was used for the measurements. The lowest concentration (%) of ammonium sulfate causing bacterial aggregation was used as a hydrophobicity index.

To determine the effect of PS preparations on bacterial viability, agarized nutrient medium (20 ml) was distributed into sterile petri dishes. On the surface of the medium, 2 ml of 0.8% agar with 0.2 ml of the bacterial suspension (10^6 cells/ml) was poured. Wells were made in the agar and filled with the pondweed polysaccharide preparations under investigation (40 μg in 0.9 NaCl solution). Equal concentrations of dextrans with the same molecular mass were used as control. The PS effect on bacterial growth was recorded only when the zone width exceeded 3 mm.

Table 1. Monosaccharide composition and molecular mass of the polysaccharide preparations from *Potamogeton perfoliatus* biomass

Polysaccharides	Molecular mass, kDa	Monosaccharide composition
n-PS1	20	Rhamnose, glucose, galactose, mannose, fucose, glucosamine
a-PS1	20	Galactose, glucuronic acid
n-PS2	6	Glucose, mannose, fucose
a-PS3	500	Rhamnose, glucose, galactose, xylose
a-PS4	500	Rhamnose, glucose, galactose
a-PS5	110	Rhamnose, glucose, mannose

The following combinations of bacterial cultures and PS preparations were tested in the experiments on pondweed PS effect on microbial growth dynamics: *X. campestris* B-610 and n-PS2 or a-PS4; *M. luteus* B-109 and a-PS4 or a-PS5; and *A. brasilense* Sp245 and n-PS2 or a-PS4. The PS concentration was 40 μg/l. Bacterial growth was monitored by OD_{420} measurements in a 1 cm cuvette. These data were used to build growth curves (the graphs of optical density depending on the cultivation time). Experimental results were treated according to the standard statistical procedures.

RESULTS AND DISCUSSION

High-molecular carbohydrate-containing material was obtained by water and water–alcohol extraction. The application of two extraction procedures allowed more complete isolation and characterization of the glycopolymers produced by the plant and penetrating into the environment. By means of gel filtration, five PS fractions (PS1–PS5) were obtained from the carbohydrate-containing extracts. In order to obtain homogeneous PS preparations, further fractionation and purification was carried out. Six PS were revealed in the pondweed polysaccharide-containing materials by means of ion-exchange chromatography; they are henceforth termed neutral (n) and acidic (a), e.g., a-PS1, n-PS1, etc.

For each of the six PS, analysis of its molecular mass and monosaccharide composition was carried out (Table 1). The data presented in the table demonstrate

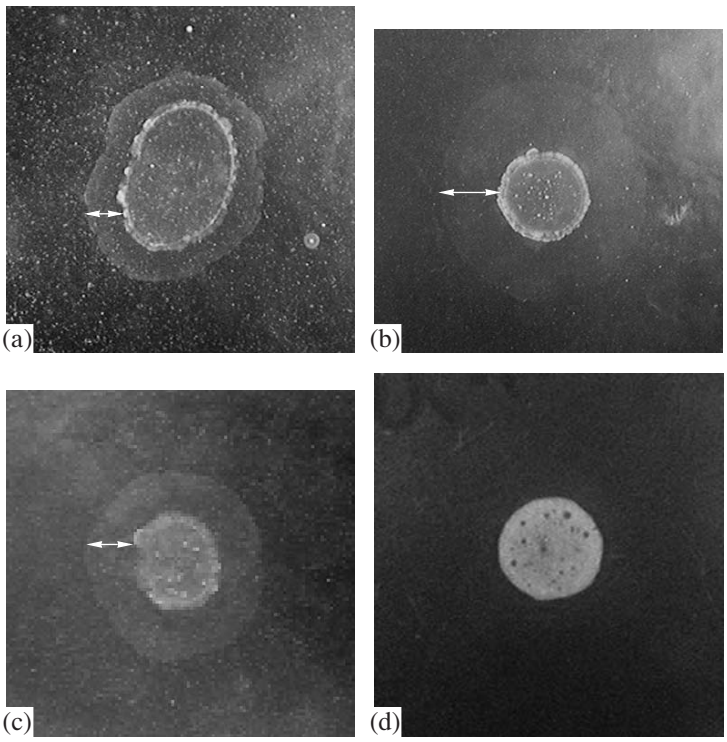

Fig. 1. *A. brasilense* Sp245 chemotaxis to *Potamogeton perfoliatus* polysaccharides: (a) preparation n-PS-1; (b) preparation n-PS-2; (c) preparation a-PS1; (d) taxis to preparation a-PS4 is absent. The arrows indicate the width of the taxis rings.

that only two PS were classified as neutral, while the other four exhibited anionic properties. In a-PS1 these properties were probably caused by the presence of uronic acid; other a-PS contained only neutral sugars, and their charge was most probably the result of the presence of noncarbohydrate anionic substituents. Although Russian researchers have recently determined the structure of *P. natans* pectin PS and demonstrated their immunomodulating activity [26], the composition of pondweed PS is poorly studied. Our data enable new insights in the composition of the water-soluble glycopolymers of this family.

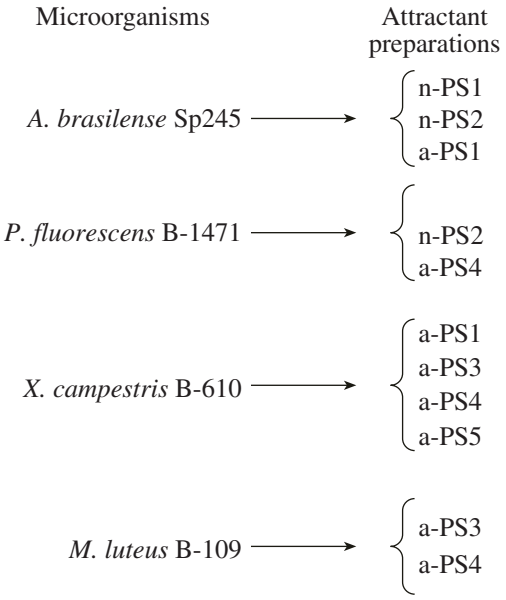

Fig. 2. Schematic representation of bacterial taxis to *Potamogeton perfoliatus* polysaccharides.

Chemotaxis can be used to test the plant–bacterial interactions. The PS concentrations used in this and other experiments were determined from the published data on glycan content in freshwater environments [19] and from preliminary experiments to establish the PS concentrations required to visualize the effect by the approaches used.

Among the strains studied, *E. coli* K12 was indifferent to all PSs. It was therefore used as a negative control. Fig. 1 demonstrates the chemotaxis of *A. brasilense* Sp245 to some of the pondweed polysaccharide preparations; the polysaccharide preparations acting as attractants to four bacterial strains are presented on Fig. 2. In our experiments, the differences in the diameter of the chemotaxis rings were insignificant. Associative bacteria (*A. brasilense* Sp245 and *P. fluorescens* B-1471) exhibited a positive reaction to neutral PS (the taxis ring was 10 to 12 mm in diameter). The phytopathogenic *X. campestris* B-160 was attracted to acidic PS (rings of 8 to 10 mm in diameter).

The positive reaction of bacteria to some pondweed PS was thus established. The mechanism of taxis to the

Table 2. Effect of *Potamogeton perfoliatus* polysaccharides on the relative hydrophobicity of microbial cell surfaces

Preparation	Hydrophobicity index, %			
	X. campestris B-610	*P. fluorescens* B-1417	*A. brasilense* Sp245	*M. luteus* B-109
Control	49.0 ± 1.5	58.0 ± 4.5	34.0 ± 1.5	65.5 ± 4.5
n-PS1	44.5 ± 1.5*	53.5 ± 3.0	43.0 ± 1.5*	41.5 ± 3.0*
n-PS2	47.5 ± 3.0	67.0 ± 1.5*	37.0 ± 3.0	46.0 ± 3.0*
a-PS1	35.5 ± 1.5*	44.5 ± 1.5*	40.0 ± 4.5*	56.5 ± 1.5*
a-PS3	32.5 ± 1.5*	58.0 ± 3.0	19.0 ± 1.5*	59.5 ± 3.0
a-PS4	34.0 ± 3.0*	53.5 ± 1.5	26.5 ± 3.0*	64.0 ± 3.0
a-PS5	44.5 ± 1.5*	62.5 ± 1.5	22.0 ± 4.5*	52.0 ± 1.5*

Note: * Indicates significant differences from the control values; the confidence interval is given for 95% reliability.

PS under investigation is yet unclear. The pondweed polysaccharide fractions may bind directly to the receptors or other cell surface structures and thus cause their conformational changes. Such interactions may change microbial hydrophobicity. The number of "transformed" receptors can in turn act as a signal for the bacterial chemotactic [27].

Hydrophobicity is one of the key characteristics of the cell surface; it is important for a number of biological processes and may have a significant effect on the formation of symbiotic and associative communities [28]. The relative hydrophobicity of bacterial surface treated with pondweed polysaccharides was investigated. The salt aggregation test was used, which makes it possible to determine primarily the contribution of the carbohydrate components in bacterial surface hydrophobicity [25]. The results presented in Table 2 demonstrate that a reliable increase in bacterial surface hydrophobicity upon treatment with PS solutions occurred in 70% of the experiments. These data indicate the binding between plant PS and bacterial surfaces; this, in turn, results in the changes in hydrophobicity. The character of these changes depends on a number of factors, including the monosaccharide composition of the PS used in the experiments. A decrease in the hydrophobicity index was observed in most of the experiments; thus, interaction with PS increased the relative hydrophobicity of bacterial surfaces. This increase in hydrophobicity was possibly caused by the presence of a deoxysugar (rhamnose) in most of the PS under investigation. The most pronounced increase in hydrophobicity was observed when xanthomonads and azospirilla were treated with a-PS3. Since increased relative hydrophobicity of bacterial surfaces usually promotes adhesion, pondweed microenvironment is possibly enriched by these microorganisms.

The effect of pondweed PS on bacterial growth on agarized medium was then investigated (Table 3). Growth of phytopathogenic *X. campestris* B-610 was found to be inhibited by n-PS1 and n-PS2 (Fig. 3a); other polysaccharides did not suppress bacterial growth. Moreover, zones of increased colony density were observed around the PS application site (Fig. 3b).

Table 3. Effect of *Potamogeton perfoliatus* polysaccharides on microbial growth on agarized media

Test culture	Preparation	Biological activity	
		Observed effect	Effect zone width, mm
X. campestris B-610	n-PS1	Growth inhibition	12.3 ± 0.1
	n-PS2	Growth inhibition	12.5 ± 0.5
	a-PS1	Growth stimulation	22.0 ± 2.0
	a-PS3	Growth stimulation	7.0 ± 0.5
	a-PS4	Growth stimulation	15.0 ± 0.2
P. fluorescens B-1417	n-PS2	Growth stimulation	5.3 ± 0.3
	a-PS4	Growth stimulation	12.0 ± 0.6
	a-PS5	Growth stimulation	3.0 ± 0.2
A. brasilense Sp245	n-PS2	Growth stimulation	25.0 ± 0.3
M. luteus B-109	a-PS4	Growth stimulation	3.0 ± 0.4

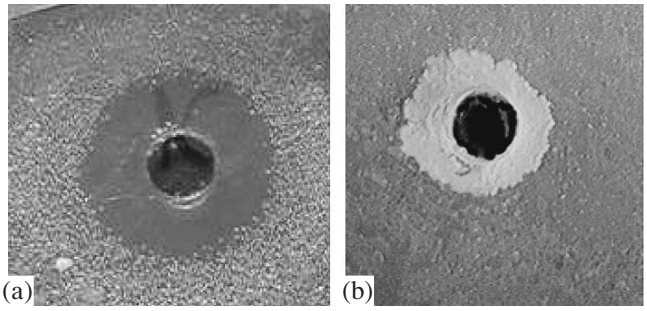

Fig. 3. Effect of the *Potamogeton perfoliatus* polysaccharide fractions on microbial growth on agarized media: (a) *X. campestris* B-610 + n-PS1; (b) *P. fluorescens* B-1471 + a-PS4.

The data presented in Table 3 mostly correlate with the previously obtained results on chemotaxis (Fig. 2). The absence of effect of a-PS3 on *M. luteus* B-109 growth and the small effect of a-PS4 are exceptional in this respect. Although these PS act as attractants for this organism, they have no effect on its surface hydrophobicity.

The effect of induction of bacterial growth is probably the result of the presence of enzymatic systems which enable these bacteria to use PS preparations as carbon sources. The mechanism of the inhibitory effect of PS on bacterial growth is poorly known. Some neutral polysaccharides are known, however, to impair the outer membrane permeability with lethal results, due to the binding of carbohydrate-containing polymers to specific receptors on bacterial cell wall. This binding probably caused significant changes in the physicochemical properties of the surface, which resulted in conformational changes in the surface proteins and therefore in their impaired functioning [29, 30].

Growth dynamics of three bacterial strains in the presence of pondweed PS revealed that a-PS4 caused a 26% increase in *X. campestris* biomass accumulation (Fig. 4a) and a 27% decrease in *A. brasilense* Sp245 biomass accumulation (Fig. 4b). No significant effect of a-PS4 on *M. luteus* growth dynamics was observed (Fig. 4c). Xanthomonads biomass accumulation was suppressed by n-PS2; this polysaccharide had a weak stimulating effect on some stages of azospirilla growth. The presence of a-PS5 decreased the time required by *M. luteus* B-109 to reach the end of the exponential growth phase (Fig. 4c). The results of these experiments are generally in good agreement with the data on chemotaxis and cell growth on agarized medium.

The results of our experiments suggest that PS participate both in the formation of the species composition of a bacterial cenosis and in the regulation of microbial biomass accumulation by selective stimulation or inhibition of certain bacterial groups in the environment. These findings also improve our understanding of the spectrum of physiological activity of glycopolymers of plant origin.

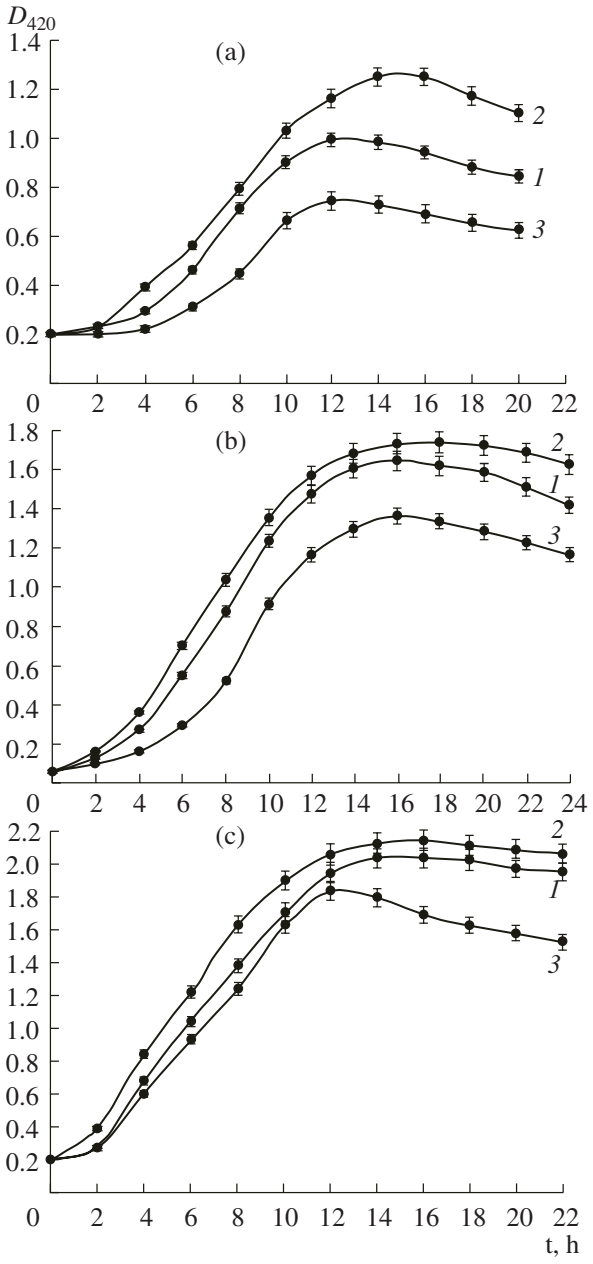

Fig. 4. Microbial growth dynamics in the presence of *Potamogeton perfoliatus* polysaccharides: (a) *X. campestris* B-610: control (*1*); a-PS4 (*2*); n-PS2 (*3*); (b) *A. brasilense* Sp245: control (*1*); n-PS2 (*2*); a-PS4 (*3*); (c) *M. luteus* B-109: control (*1*); a-PS4 (*2*); a-PS5 (*3*).

ACKNOWLEDGMENTS

The work was partially supported by the Russian Foundation for Basic Research (project no. 05-04-48123) and by the grant of the President of the Russian Federation no. NSh 6177.2006.4.

REFERENCES

1. Chang, H., Yang, X., Fang, Y., Pu, P., Li, Z., and Rengel, Z., In situ Nitrogen Removal from the Eutrophic Water by Microbial–Plant Integrated System, *J. Zhejiang. Univ. Science, B*, 2006, vol. 7, no. 7, pp. 521–531.
2. Van Donk, E. and van de Bund, W.J., Impact of Submerged Macrophytes Including Charophytes on Phyto- and Zooplankton Communities: Allelopathy Versus Other Mechanisms, *Aquat. Bot.*, 2002, vol. 72, nos. 3-4, pp. 261–274.
3. Khirnaya, A.N. and Fedii, S.P., Water Quality Assessment by Amino Acid Content in the Biochemical Composition of Soft Aquatic Vegetation, in *Samoochishchenie i bioindikatsiya zagryaznennykh vod* (Self-Purification and Bioindication of Contaminated Waters), Moscow: Nauka, 1980.
4. Ostroumov, S.A., Biological Mechanism of Self-Purification in Natural Water Bodies and Flows: Theory and Applications, *Usp. Sovrem. Biol.*, 2004, vol. 124, no. 5, pp. 429–442.
5. Fisher, M.M., Klug, J.L., Lauster, G., Newton, M., and Triplett, E.W., Effects of Resources and Trophic Interactions on Freshwater Bacterioplankton Diversity, *Microb. Ecol.*, 2000, vol. 40, pp. 125–138.
6. Carpenter, S.R. and Lodge, D.M., Effects of Submersed Macrophytes on Ecosystem Processes, *Aquat. Bot.*, 1986, vol. 26, pp. 341–370.
7. Carter, V., Rybicki, N.B., and Hammerschlag, R., Effects of Submersed Macrophytes on Dissolved Oxygen, pH, and Temperature under Different Conditions of Wind, Tide and Bed Structure, *J. Freshwat. Ecol.*, 1991, vol. 6, pp. 121–133.
8. Caffrey, J.M. and Kemp, W.M., Seasonal and Spatial Patterns of Oxygen Production, Respiration and Root-Rhizome Release in *Potamogeton perfoliatus* L. and *Zostera marina* L., *Aquat. Bot.*, 1991, vol. 40, no. 1, pp. 109–128.
9. *Flora i produktivnost' pelagicheskikh i litoral'nykh fitotsenozov vodoemov basseina Volgi* (Flora and Productivity of Pelagic and Littoral Phytocenoses in the Volga Basin Reservoirs), Leningrad: Nauka, 1990.
10. Einor, L.O., *Makrofity v ekologii vodoema* (Macrophytes in Ecology of Water Bodies), Moscow: IVP RAN, 1992.
11. Neundorfer, J.V. and Kemp, W.M., Nitrogen Versus Phosphorus Enrichment of Brackish Waters: Responses of the Submersed Plant *Potamogeton perfoliatus* and Its Associated Algal Community, *Mar. Ecol.: Proc. Ser.*, 1993, vol. 94, pp. 71–82.
12. Lukina, L.F. and Smirnova, N.N., *Fiziologiya vysshikh vodnykh rastenii* (Physiology of Aquatic Higher Plants), Kiev: Naukova dumka, 1988.
13. Burkovskii, I.V. and Stolyarov, A.P., Structural and Functional Differentiation and Integration in a Marine Coastal Ecosystem, *Usp. Sovrem. Biol.*, 2000, vol. 120, no. 5, pp. 433–440.
14. Bukharin, O.V. and Nemtsov, N.V., Symbiotic Interactions of Hydrobionts and their Reaction to Antropogenic Impact, *Izv. Samarskogo Nauchnogo Tsentra RAN*, 2003, vol. 1, pp. 53–65.
15. Xie, C.H. and Yokota, A., *Azospirillum oryzae* sp. nov., a Nitrogen-Fixing Bacterium Isolated from the Roots of the Rice Plant *Oryza sativa, Int. J. Syst. Evol. Microbiol.*, 2005, vol. 55, no. 4, pp. 1435–1438.
16. Zvyagintsev, D.G., Dobrovol'skaya, T.G., and Lysak, L.V., Plants as Formation Centers of Bacterial Communities, *Obshchaya Biologiya*, 1993, vol. 54, no. 2, pp. 21–25.
17. Gallagher, J.L., Wolf, P.L., and Pfeiffer, W.J., Rhizome and Root Growth Rates and Cycles in Protein and Carbohydrate Concentrations in Georgia (USA) *Spartina alterniflora, Am. J. Bot.*, 1984, vol. 71, pp. 165–169.
18. Usov, A.I. and Elashvili, M.Ya., Research on Macrophyte Polysaccharides, *Bioorg. Khim.*, 1991, vol. 17, no. 6, pp. 839–848.
19. Tien, C.-J., Krivtsov, V., Levado, E., Sigee, D.C., and White, K.N., Occurrence of Cell-Associated Mucilage and Soluble Extracellular Polysaccharides in Rostherne Mere and Their Possible Significance, *Hydrobiol.*, 2002, vol. 485, pp. 245–252.
20. *Manual of Methods for General Bacteriology*, Gerhardt, P. et al., Eds., Washington: Amer. Soc. Microbiol., 1918 [Russ. Transl. Moscow: Mir, 1984].
21. Katanskaya, V.M., *Vysshaya vodnaya rastitel'nost' kontinental'nykh vodoemov SSSR* (Higher Aquatic Vegetation of the Continental Reservoirs of the USSR), Leningrad: Nauka, 1981.
22. Polle, A.Ya., Ovodova, R.G., and Popov, S.V., Isolation and Characterization of the Polysaccharides of Costmary, Coltsfoot, and Burdock, *Khim. Rastit. Syr'ya*, 1999, no. 1, pp. 33–38.
23. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., Colorimetric Method for Determination of Sugars and Related Substances, *Anal. Chem.*, 1956, vol. 23, no. 3, pp. 350–356.
24. Armstrong, J.B., Adler, J., and Dahl, M.M., Nonchemotactic Mutants of *Escherichia coli, J. Bacteriol.*, 1967, vol. 93, pp. 390–398.
25. Dillon, J.K., Fuerst, J.A., Hayward, A.C., and Davis, G.H.G., A Comparison of Five Methods for Assaying Bacterial Hydrophobicity, *J. Microbiol. Methods*, 1986, no. 6, pp. 13–19.
26. Popov, S.V., Popova, G.Y., Paderin, N.M., Koval, O.A., Ovodova, R.G., and Ovodov, Y.S., Preventive Anti-Inflammatory Effect of Potamogetonan, a Pectin from the Common Pondweed *Potamogeton natans, Phytother. Res.*, 2007, in press.
27. Brezgunov, V.N., Zaval'skii, L.Yu., and Lazarev, A.V., Bacterial Chemotaxis, *Usp. Mikrobiol.*, 1989, vol. 23, pp. 3–28.
28. Nikovskaya, G.N., Gordienko, A.S., and Globa, L.I., Hydrophilic and Hydrophobic Properties of Microorganisms under Different Cultivation Conditions, *Mikrobiologiya*, 1985, vol. 58, no. 3, pp. 448–451.
29. Gil1, G., del Monaco, S., Cerrutti, P., and Galvagno, M., Selective Antimicrobial Activity of Chitosan on Beer Spoilage Bacteria and Brewing Yeasts, *Biotechnol Lett.*, 2004, vol. 26, pp. 569–574.
30. Vaara, M., Agents That Increase the Permeability of the Outer Membrane, *Microbiol. Rev.*, 1992, vol. 56, no. 3, pp. 395–411.