Quantification of two forms of green sulfur bacteria in their natural habitat using bacteriochlorophyll fluorescence spectra

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ABSTRACT

Detection of phototropic organisms in their natural habitat using optical instruments operating under water is urgently needed for many tasks of ecological monitoring. While fluorescence methods are widely applied nowadays to detect and characterize phytoplankton communities, the techniques for detection and recognition of anoxygenic phototrophs are considered challenging. Differentiation of the forms of anoxygenic green sulfur bacteria in natural water using spectral techniques remains problematic. Green sulfur bacteria could be found in two forms, green-colored (containing BChl *d* in pigment compound) and brown-colored (containing BChl *e*), have the special ecological niche in such reservoirs. Separate determination of these microorganisms by spectral methods is complicated because of similarity of spectral characteristics of their pigments. We describe the novel technique of quantification of two forms of green sulfur bacteria directly in water using bacteriochlorophyll fluorescence without pigment extraction. This technique is noninvasive and could be applied in remote mode in the water bodies with restricted water circulation to determine simultaneously concentrations of two forms of green sulfur bacteria in their natural habitat.

Keywords: absorbance, fluorescence, green sulfur bacteria, anoxygenic phototrophic microorganisms, bacteriochlorophyll, carotenoids, natural water.

1. INTRODUCTION

In the recent 10 years there were found several relic water reservoirs with the signs of meromixis on the Karelian coast of the White Sea, which were formed as a result of postglacial isostatic uplift of the sea shores.[1-4] The forming of the lakes with stratified water structure near the White Sea is one of the least studied processes.[3] These reservoirs have an upper freshened layer and the bottom salty anaerobic layer. A chemocline with sharp physical and chemical gradients, including redox potential, exists at the border between the salty aerobic and anaerobic layers. The formation of large population of anoxygenic bacteria around the redox zone was described.[5-6]

Detection of phototropic organisms in their natural habitat is needed in many tasks of ecological monitoring and can be performed online using optical instruments operating being submerged under water. Fluorescence methods to detect and characterize chromophoric dissolved organic matter (CDOM) and phytoplankton communities are widely applied nowadays, while detection and recognition of anoxygenic phototrophic microorganisms is considered challenging. All types of natural water under UV excitation manifests fluorescence of chromophoric dissolved organic matter (CDOM)[7-8], its fluorescent components[9] and transformation changes due to microorganisms[10]. CDOM demonstrates so called humic-type emission band in the blue region which emission wavelength and intensity depend on the source of organic material[7-10], temperature and UV irradiation[11], excitation wavelength and laser pulse power used for spectra acquisition[10-12]. Oxygenic phototrophic microorganisms in natural waters are presented by algae and cyanobacteria with dominating chlorophyll *a* as a photosynthetic pigment. Fluorescence of chlorophyll *a* is widely used to study phytoplankton[13 -15]and cyanobacteria[16]. Anoxygenic phototrophic microorganisms in water reservoirs include green and purple bacteria with apparently distinct absorption bands. However differentiation of forms of green sulfur bacteria in natural water using spectral techniques is challenging. Green sulfur bacteria could be found in two forms, green-colored (containing BChl *d* in pigment compound) and brown-colored (containing BChl *e*), have the special ecological niche in such reservoirs.

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Separate determination of these microorganisms by spectral methods is complicated because of the similarity of spectral characteristics of their pigments.

Noninvasive fluorescence techniques for monitoring of anoxygenic bacteria in are particularly in demand for diagnostics of microbial communities inside the water bodies with restricted water circulation. The aim of this work is to find spectral algorithms which could be applied in remote mode to determine contributions of two forms of green sulfur bacteria in microbial community directly in their natural habitat.

2. GREEN SULFUR BACTERIA AND THEIR PHOTOSYNTHETIC PIGMENTS

2.1. Green sulfur bacteria

Green sulfur bacteria belong to anoxygenic phototrophs, the most ancient group of phototrophic organisms on the Earth. According to molecular biology, these phototrophs appeared about 3.5 billion years ago[17]. Green sulfur bacteria have photochemical reaction centers for converting absorb light energy into chemical.[18] Scientific interest in the study of green sulfur bacteria is determined by the simple organization of their photosystem, which makes them suitable models for biochemical and biophysical research.

Green sulfur bacteria are widely distributed in the water reservoirs, usually in an environment containing hydrogen sulfide (ponds, sea lagoons, lakes with restricted water circulation) because it is necessary for their photosynthesis.[19] Green sulfur bacteria are mainly immobile. These bacteria conduct photosynthesis, but do not produce oxygen. As photosynthetic pigments they contain various bacteriochlorophylls (BChls) and carotenoids. Due to this they are able to utilize the light at wavelengths not absorbed by plants, algae or cyanobacteria. Green sulfur bacteria absorb photons very effectively with the help of the chlorosome antenna complexes and perform photosynthesis in extreme low-light environments.

There are two morphologically similar species of green sulfur bacteria but with different coloration of the cells: green (with bacteriochlorophyll *c* and *d* and carotenoid chlorobactin) and chocolate brown (with bacteriochlorophyll *e* and carotenoid isorenieratin)[20]. Isorenieratin absorbs light at longer wavelengths compared to chlorobactin, so the brown species of green sulfur bacteria dominate at depths, where the green light is predominant.[21]

2.2. Photosynthetic pigments of green sulfur bacteria and spectral properties

Photosynthetic bacteria are characterized by chromosome pressed against the inner side of the plasma membrane associated with the cytoplasmic membrane.[22-23] During the process highly organized photosynthetic membrane units convert light into biochemical energy with high efficiency.[24] Chlorosomes filled molecules of BChl *c*, *d* or *e* in a highly aggregated state. Green colored bacteria contain mainly bacteriochlorophyll *c* or *d*, whereas other species have orange or brown in color due to the high content of carotenoids.

Bacteriochlorophylls absorb light quanta of certain energy. Typically, the adsorption properties of the pigments are studied in vitro into organic solvents by extraction. By the main characteristics of the absorption spectra in organic solvents BChl chromophores can be divided into six main types, which are designated by Latin letters *a* to *e* and *g*[25]. BChl seventh structure, which is designated by the letter *f*, is known, but was obtained by extraction of any kind of bacteria. BChl *f* first isolated from the mutant strain. But since it is similar to BChl *e*, there is speculation that it may exist.

BChl extraction may be concentrated from cell cultures or cell fractions with the use of organic solvents which water is removed before the pigments dissolve. For this purpose, a mixture of methanol, acetone or pyridine and water can similarly use these solvents in combination with each other or with diethyl ether, petroleum ether or carbon tetrachloride. Before you make the BChl into an organic solvent, it must be remembered that all types of chlorophyll are very unstable pigments, the stability of which is affected by external factors (light acidity, the presence of oxygen), so in order to prevent degradation of the pigment molecule, all actions necessary to carry out in the dark or in the dim light.

The main photosynthetic pigments of green sulfur bacteria present absorption peaks in the region 745-755 nm, BChl *d* (725-745 nm), the BChl *e* (710-725 nm)[26]. Also, green bacteria are characterized by a small number of BChl *a* (805-812 nm), which is part of the reaction centers.

3. OBJECTS AND METHODS

3.1. Green sulfur bacteria cultures

The objects of the study were anoxygenic phototrophic bacteria communities from three stratified lakes located on the coast of the Kandalaksha Bay of the White Sea: Trekhzvetnoe (Tricolor) Lake, Lagoon at Zeleny ("Green") Cape and Elovoe Lake. In August and September 2014 the expedition was held on the shores of the Kanadalaksha Bay of the White Sea. Depth profiles of physical and chemical characteristics of the water column and water samples were obtained for each lake. Freshly drawn water samples were hermetically sealed in vials for storage and further culturing of anoxygenic phototrophic bacteria. Cultivation was carried out anaerobically for several months in the climate chamber with illumination at 2000 Lux and the temperature 20-25°C.

3.2. Extractions of green sulfur bacteria

For pigment extraction the bacterial cultures were pelleted by centrifugation and dissolved in a mixture of acetone and methanol (7: 2). After extraction preparation the tubes were hermetically sealed, wrapped into aluminum foil and placed in refrigerator for several days. The mixture then was centrifuged again and the supernatant was used for spectral measurements.

3.3. Spectral measurements

The fluorescence spectra of the bacterial cultures were recorded with the luminescence spectrometer Solar CM2203 in standard quartz cuvettes with a path length of 10 mm. For all the samples fluorescence emission spectra were recorded using excitation wavelengths λ_{ex} = 390 and 440 nm, and fluorescence excitation spectra were registered at wavelengths λ_{em} = 770 and 815 nm. The absorption spectra of the samples were measured with a spectrophotometer Unico. Absorbance spectra of green sulfur bacteria cultures were recorded relative to a 1% solution of NaCl (sodium chloride) and $Na_2S_2O_3$ (sodium thiosulfate) in the spectral range of 200-1000 nm. Absorbance spectra of pigments extractions were recorded relative to a mixture of acetone and methanol in a ratio of 7: 2 in the same spectral range.

4. RESULTS AND DISCUSSION

4.1. Absorption spectra of green sulfur bacteria cultures

Absorbance spectra of living cells of green sulfur bacteria with different pigmentation in anaerobic aqueous medium are given in Figure 1.

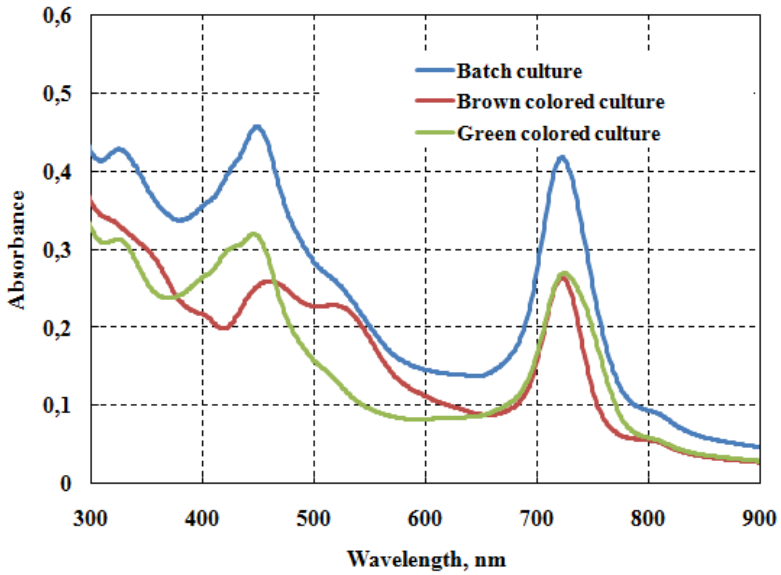

Figure 1. Absorbance spectra of cultures of green sulfur bacteria with different pigmentstion.

Green colored forms of sulfur bacteria are characterized by broad absorption band with a maximum at 450 nm corresponding to the absorption of BChl *d*. For brown colored green sulfur bacteria the characteristic absorption band is located at 465 nm corresponding to the absorption peak of BChl *e* and at 525 nm, the wavelength of absorption for carotenoids. The height of the peaks is dependent on the concentration of sulfur bacteria cells in the culture.

The absorption spectra of cumulative culture of green sulfur bacteria represent the total spectrum absorbance of green colored and brown colored and cultures with all the characteristic absorption bands, indicating that the simultaneous presence of such a culture of molecules BChl *d*, and *e*. The height of the peaks depends on the cell concentration in the solution; the ratio of absorption band varies depending on the ratio of sulfur bacteria cells, having a green or brown. The range of cultural accumulation of green sulfur bacteria in the wavelength region has a broad absorption band.

4.2. Absorbance of green sulfur bacteria extracts

The absorption spectra of a single color extracts sulfur bacteria contain two narrow peaks with maxima in the 650-660 nm wavelength region and short - 420-480 nm. In the long-wavelength region, both cultures have a maximum of 555 nm, however, the optical density for green colored sulfur bacteria was higher in comparison with the optical density of brown colored sulfur bacteria with the same concentration of cells in the original culture.

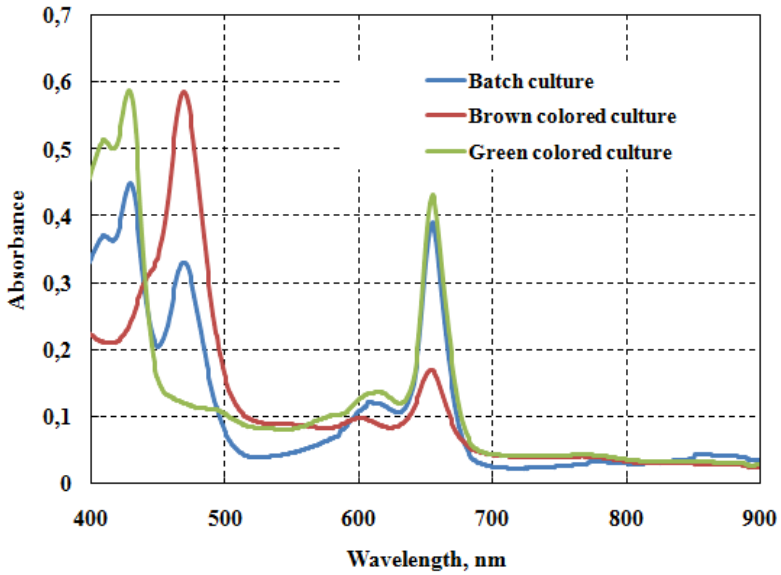

Figure 2. Absorbance spectra of pigment extractions for the of cultures of green sulfur bacteria with different pigmentstion.

Short-wave absorption band of extracts of green colored, brown colored and green sulfur bacteria has fundamental differences. Green colored extracts of green sulfur bacteria have an absorption band with a maximum at 430 nm corresponding to the absorption of light BChl *d*. Brown colored extracts of green sulfur bacteria have an absorption band with a peak wavelength of 470 nm corresponding to the absorption of light BChl *e*. The optical density depends on the concentration of cells of green sulfur bacteria in the original cultures.

To extract enrichment cultures of green sulfur bacteria characteristic features inherent green colored, brown colored and green sulfur bacteria at the same time. The optical density of the long-wave maximum as compared with pure cultures of bacteria of the same one color cell concentration in the initial solution. In the short-wavelength region of the absorption spectrum of cumulative culture contains two peaks corresponding to the absorption of light BChl *d*, and *e*.

4.3. Fluorescence of green sulfur bacteria

Fluorescence emission spectra of green sulfur bacteria crops were recorded at excitation wavelengths λex = 390 and 440 nm. The emission spectra of all three crops (funded, and green colored, brown colored) found two fluorescence maximum: in the area of 740-770 nm and at a wavelength of 815 nm corresponding to the emission of molecules of BChl *c*, *d* and *e*.

The position of the second maximum in the area of 815 nm did not depend on the culture and the presence of pigments. The first maximum fluorescence of green colored green sulfur bacteria was located at a wavelength of 765 nm corresponding to the emission of the fluorescence pigment BChl d. This maximum fluorescence of brown colored culture of green sulfur bacteria was recorded at a wavelength of 745 nm corresponding to the emission of light BChl *e*.

In batch culture green sulfur bacteria are present simultaneously two pigment - BChl *d* and *e*, whereby corresponding maximum fluorescence of the culture is at a wavelength of 758 nm. The position of the maximum ratio has been evaluated and green colored, brown colored green sulfur bacteria: in this culture there is (61,9 ± 4,8)% green colored green sulfur bacteria.

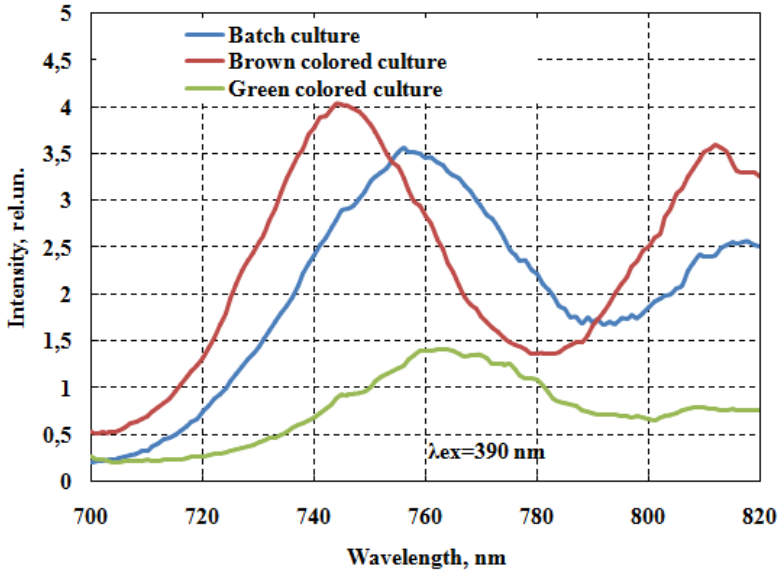

Figure 3. Fluorescence emission spectra for cultures green sulfur bacteria measured in the spectral range of bacteriochlorophylls (λ_{ex}=390 nm).

Fluorescence excitation spectra were recorded at an emission wavelength λem = 770 and 815 nm. They are characterized by excitation peaks corresponding to the absorption bands of photosynthetic pigments in the culture (Bchl, carotenoids).

The long-wavelength maximum located at a wavelength of 720 nm irrespective of the type of the measured culture. Green colored culture has a broad maximum at 450 nm (BChl *d*), brown colored - two peaks, 460 (BChl *e*) and 530 nm (carotenoids). Fluorescence excitation spectra of cumulative culture of green sulfur bacteria contains all the peaks characteristic of green colored and brown colored and cultures. The shapes of the excitation spectra are similar to the spectra of the optical density of the bacterial cultures.

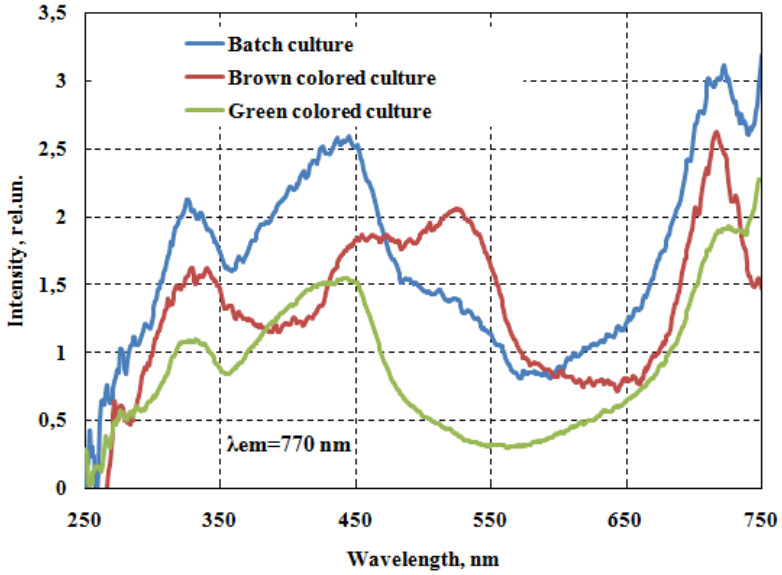

Figure 4. Bacteriochlorophyll fluorescence excitation spectra for the cultures of green sulfur bacteria (λ_{em}=770 nm).

4.4. Bacteriochlorophyll fluorescence band deconvolution into individual spectral components

Usually, natural water samples and enrichment cultures there are different kinds of green sulfur bacteria, however, depending on their ratio is shifted from the maximum fluorescence wavelengths, for green-colored (765-770 nm) and brown colored (735-740) species separately. This fact makes it possible to assess the position of the maximum ratio of different species in water samples. The expansion of the maximum fluorescence emission spectrum in the 740-770 nm into two Gaussian (with peaks at wavelengths of 740 and 765 nm) and calculation areas under curves was evaluated relative share content of green-colored and brown-colored green sulfur bacteria in the water samples.

5. CONCLUSIONS

We sum up the main results of the spectroscopic research:

- Absorbance spectra of cultures of green sulfur bacteria in aqueous medium contain absorption bands by chlorophyll *a*, bacteriochlorophyll *d* and *e* and various carotenoids.
- The samples of water with green sulfur bacteria demonstrated two distinct peaks of bacteriochlorophyll fluorescence (1) with maximum at wavelength λ = 740-770 nm corresponding to emission of bacteriochlorophyll *d* or *e* and (2) at 820 nm with constant wavelength of maximum attributed as emission of bacteriochlorophyll *a*.
- The main bacterial chlorophyll of green sulfur bacteria is bacteriochlorophyll *d* in green-colored cultures and bacteriochlorophyll *e* in brown-colored cultures.
- The position of emission maximum of the first bacteriochlorophyll peak is gradually shifted with alteration of ratio of two bacterial forms with different pigmentation.

On the basis of our findings we propose the following algorithm of calculation of the relative proportion of green and brown colored green sulfur bacteria in water. In order to separate the contributions of various groups of phototrophic bacteria is necessary to deconvolute the fluorescence band in the 740-770 nm into two components and calculate the ratio of the integral intensities for different bacteriochlorophylls.

The practical importance of the work is determined by the development of a method for environmental monitoring of water bodies with the violations of the circulation of water – separated from the sea dams bays, artificial reservoirs and process water tanks, which is especially important with respect to the Arctic area.

REFERENCES

[1] Krasnova, E.D., Pantyulin, A.N., Matorin, D.N., Todorenko, D.A., Belevich, T.A., Milyutina, I.A., Voronov, D.A., "Cryptomonat alga *Rhodomonas* sp. (*Cryptophyta, Pyrenomonadaceae*) bloom in the redox zone of the basins separating from White Sea," Microbiology 83 (3), 270-277 (2014).

[2] Vinogradov, D., Varlamov, S., Volovich, N., Kuznetsov, V., Grigoryeva, A., Mardashova, M., Krasnova, E., "Hydrological and spectrophotometry research on Kislo-Sladkoye lake," EARSeL eProceedings 14 (S1), 55–62 (2015).

[3] Krasnova, E.D., Pantyulin, A.N., Belevich, T.A., Voronov, D.A., Demidenko, N.A., Zhitina, L.S., Ilyash, L.V., Kokryatskaya, N.M., Lunina, O.N., Mardashova, M.V., Prudkovsky, A.A., Savvichev, A.S., Filippov, A.S., Shevchenko, V.P., "Multidisciplinary studies of the separating lakes at different stage of isolation from the White Sea performed in March 2012," Oceanology 53 (5), 714-717 (2013).

[4] Krasnova, E., Voronov, D., Frolova, N., Pantyulin, A., Samsonov, T., "Salt lakes separated from the White Sea," EARSeL eProceedings 14 (S1), 8–22 (2015).

[5] Savvichev, A.S., Lunina, O.N., Rusanov, I.I., Zakharova, E.E., Veslopolova, E.F., Ivanov, M.V., "Microbiological and isotopic geochemical investigation of Lake Kislo-Sladkoe, a meromictic water body at the Kandalaksha Bay shore (White Sea)," Microbiology 83 (1), 56-66 (2014).

[6] Kharcheva, A.V., Krasnova, E.D., Voronov, D.A., Patsaeva, S.V., "Spectroscopic study of the microbial community in chemocline zones of relic meromictic lakes separating from the White Sea," Proceedings of SPIE – The International Society for Optical Engineering 9448, (2015).

[7] Blough, N.V., Del Vecchio, R. [Hansel, D.A., Carlson, C.A. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter], "Chromophoric DOM in the coastal environment". Academic Press, San Diego, California, 509–546 (2002).

[8] Trubetskoj, O.A., Trubetskaya, O.E., Richard, C., "Photochemical activity and fluorescence of aquatic humic matter," Water Resour. 36, 518-524 (2009).

[9] Patsayeva, S., Reuter, R., "Spectroscopic study of major components of dissolved organic matter naturally occurring in water," Proceedings of SPIE – The International Society for Optical Engineering 2586, 151-160 (1995).

[10] Khundzhua, D., Patsaeva, S., Terekhova, V., Yuzhakov, V., "Spectral characterization of fungal metabolites in aqueous medium with humus substances," Journal of Spectroscopy (Hindawi Publishing Corporation) (Article ID 538608), 1–7 (2013).

[11] Patsayeva, S. V., Fadeev, V. V., Filippova, E. M., Yuzhakov V.I., "Temperature and laser ultraviolet radiation influence on luminescence spectra of dissolved organic matter," Moscow University Phys. Bull 32 (6), 71–75 (1991).

[12] Patsayeva, S.V., Fadeev, V.V., Filippova, E.M., Yuzhakov, V.I., "The fluorescence saturation effect of dissolved organic matter," Moscow University Phys. Bull 33 (5), 38-42 (1992).

[13] Fadeev, V.V., Gorbunov, M.Y., Gostev, T.S., "Studying photoprotective processes in the green alga chlorella pyrenoidosa using nonlinear laser fluorimetry," Journal of Biophotonics 5 (7), 502–507 (2012).

[14] Lenbaum, V., Bulychev, A., Matorin, D., "Effects of far red light on the induction changes of prompt and delayed fluorescence and the redox state of p700 in scenedesmus quadricauda," Russian Journal of Plant Physiology. 62(2), 210–210 (2015).

[15] Terekhova, V.A., Kydralieva, K.A., Matorin, D.N., Lisovitskaya, O.V., Yurishcheva, A.A., "Biological activity of nanocomposite detoxicant in biotest-systems," 8, 4–14, (2014).

[16] Vershubskii, A., Mishanin, V., Tikhonov, A., "Modeling of the photosynthetic electron transport regulation in cyanobacteria," Biochemistry Supplemental Series A. 8(3), 262–278 (2014).

[17] Gorlenko, V.M., Rozhnov, S.V. [Photosynthesis early evolution problems], "Geo-biological processes in the past," PIN RAS, Moscow, (2011).

[18] Badalamenti, J.P., Torres, C.I., Krajmalnik-Brown, R., "Light-responsive current generation by phototrophically enriched anode biofilms dominated by green sulfur bacteria," Biotechnology and Bioengineering 110 (4), 1020-1027 (2013).

[19] Krasnova, E.D., Kharcheva, A.V., Milyutina, I.A., Voronov, D.A., Patsaeva, S.V., "Study of microbial communities in redox zone of meromictic lakes isolated from the White Sea using spectral and molecular methods," Journal of the Marine Biological Association of the United Kingdom, 95 (8), 1579-1590 (2015).

[20] Lunina, O.N., Savvichev, A.S., Kuznetsov, B.B., Pimenov, N.V., Gorlenko, V.M., "Anoxygenic Phototrophic Bacteria of the Kislo-Sladkoe Stratified Lake (White Sea, Kandalaksha Bay)," Microbiology 82 (6), 815–832 (2013).

[21] Galchenko, V.F. [Proceedings of the Winogradsky Institute of Microbiology, Vol. 15: Photosynthetic organisms], "Winogradsky Institute of Microbiology, RAS, MAKS Press, Moscow", (2010).

[22] Olson, J.M., "Chlorophyll organization and function in green photosynthetic bacteria," Photochemistry and Photobiology 67 (1), 61-75 (1998).

[23] Orf, G., Blankenship, R.E., "Chlorosome antenna complexes from green photosynthetic bacteria," Photosynthesis Research 116 (2-3), 315-331 (2013).

[24] Adams, P.G., Cadby, A.J., Robinson, B., Tsukatani, Y., Tank, M., Wen, J., Blankenship, R.E., Bryant, D.A., Hunter, C. N., "Comparison of the physical characteristics of chlorosomes from three different phyla of green phototrophic bacteria," Biochimica et Biophysics Acta 1827, 1235-1244 (2013).

[25] Oelze, J., "Analysis of bacteriochlorophylls," Methods in Microbiology 18, 257-284 (1985).

[26] Kharcheva, A.V., Meschankin, A.V., Lyalin, I.I., Krasnova, E.D., Voronov, D.A., Patsaeva, S.V., "The study of coastal meromictic water basins in the Kandalaksha Gulf of the White Sea by spectral and physicochemical methods," Proceedings of SPIE – The International Society for Optical Engineering 9031, (2014).