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OPTICS AND SPECTROSCOPY. LASER PHYSICS

Quantification of Chlorosomal Bacteriochlorophylls Using Absorption Spectra of Green Sulfur Bacteria in Natural Water

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Abstract—A non-extraction method for determining the concentration of chlorosomal bacteriochlorophylls (Bchl), photosynthetic pigments of green sulfur bacteria has been developed and tested. The method is based on the measurement of optical density spectra of microorganisms directly in natural water and is aimed at simplifying Bchl concentration measurements, reducing the measurement time and eliminating toxic solvents from the experiments. Comparison of the results of Bchl concentration determination by the traditional method based on the measurement of absorption spectra of pigment extracts and the new method that is based on the measurement of long wavelength absorption band of Bchl in bacterial cells in natural water showed a high correlation coefficient from 0.9866 to 0.9991 for different series of samples. The depth distribution profiles of Bchl concentration obtained from the absorption spectra of natural water in some meromictic reservoirs of the White Sea region in September 2019 and Lake Mogilnoye (Barents Sea) in June 2019 are presented.

Keywords: spectroscopy, bacteriochlorophyll, green sulfur bacteria, concentration, pigments.

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INTRODUCTION

Spectral-optical methods are widely used in oceanographic and limnological research. The spectral characteristics of aquatic organisms containing chlorophyll (Chl) have been fairly well studied [1, 2], while the optical properties of photosynthetic pigments of anoxygenic phototrophic bacteria, "bacteriochlorophyll (Bchl)," are still poorly studied and are little used in practice due to the difficulty of water sampling, their transportation to the laboratory, the complexity of cultivating anoxygenic phototrophs, as well as the difference between cultured strains and natural ones.

In the area of the Kandalaksha Bay of the White sea, there are several reservoirs at different stages of isolation from the White Sea [3–6]. In these reservoirs on the border between the upper oxygen-reach and lower oxygen-free layers, favorable conditions have developed for specific microorganisms, such as green sulfur bacteria (GSB) [7, 8]. The main photosynthetic pigments of GSB are chlorosomal bacteriochlorophylls Bchl *c*, Bchl *d*, and Bchl *e*. Due to the specific conditions necessary for the life of GSB, their presence in the water indicates the presence of hydrogen sulfide accumulation of the reservoir. Information about the dynamics of the position of the hydrogen sulfide layer in the reservoir makes it possible to predict what will happen to this reservoir in the future.

This work concerns the improvement and practical application of the spectral method for determining the concentration of chlorosomal Bchl of green sulfur bacteria in water samples without extracting pigments. The empirical formula [9] obtained by Overmann and Tilzer in 1989 has been widely used to determine the concentrations of chlorosomal Bchl. This formula includes the optical densities of bands at

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451 and 663 nm in the absorption spectrum of Bchl in acetone extract. However, the preparation of extracts from water samples with GSB is complicated by the need for pre-filtration or centrifugation of the sample to increase the concentration of pigments, using toxic organic solvents such as acetone and methanol, as well as the need to keep the extract for at least 1 day in a cold dark place for complete extraction of Bchl. In some cases, it is necessary to centrifuge the resulting extracts to precipitate scattering particles. This work is aimed at simplifying the measurement of Bchl concentration, reducing the measurement time, and reducing the accompanying risks associated with the use of toxic solvents.

1. OBJECTS AND METHODS

In the development of the new method for determining the concentration of chlorosomal Bchl we used water samples taken in March 2018 from several meromictic reservoirs separating from the White Sea: the lagoon on Cape Zeleny, Lake Bolshie Khruslomeny and Lake Trekhtsvetnoe which contain green- and brown-colored GSB. The method was tested in different seasons of 2018–2019 using samples taken from the same reservoirs, as well as from Lake Elovoe (coast of the White Sea) and Lake Mogilnoye (Kil'din island in the Barents Sea).

Samples were filtrated using cellulose acetate filters with a pore diameter of 0.22 μm or polyethylene terephthalate filters with a pore diameter of 0.2 μm. To determine the concentrations of Bchl using spectrometric formula, extracts were prepared from the water samples containing GSB. A mixture of acetone and ethanol in a ratio of 7 to 2 was chosen as the solvent.

The optical density spectra of the samples were measured in the spectral range of 300–900 nm with a resolution of 1 nm using standard photometry cuvettes with an optical path length of 1, 2, or 3 cm and Solar PB2201 or PV1251 spectrophotometers. For further calculations, all optical densities were reduced to an optical path length of 1 cm.

2. ABSORPTION SPECTRA OF GREEN SULFUR BACTERIA IN WATER

Figure 1 presents examples of absorbtion spectra of water samples with green- and brown-colored GSB. The absorption spectrum of the sample from Lake Trekhtsvetnoe shows two pronounced absorption bands of Bchl *d* of green-colored GSB: a short wavelength band with a maximum in the range of 445–455 nm and a long wavelength band with a maximum in the range of 725–735 nm. The green-colored GSB are characterized by a relatively low content of carotenoid chlorobactene (almost 4 times less than that the content of carotenoid isorenieratene in brown-colored GSB); therefore, in the absorption spectra of water with green-colored GSB, the band due to the chlorobactene absorption is practically not observed. The absorption spectrum of the sample from the lagoon on Cape Zeleny also shows two pronounced absorption bands of Bchl *e* of brown-colored GSB with maxima in the ranges of 450–460 nm and 715–725 nm, as well as an absorption band of carotenoid isorenieratin with a maximum in the range of 535–545 nm.

Because of light scattering, the absorption bands of carotenoids are poorly distinguishable in the absorption spectra of all the studied series of water samples with GSB. The main source of light scattering in the studied water samples are particles with a diameter of less than 2 μm, including heterotrophic and photoautotrophic bacteria and the tiny eukaryotic cells [10]. The scattering particles also include GSB themselves with dimensions $(0.7–1.0) \times (0.8–1.6)$ μm [11]. The scattering of light by such particles is described by the Mie theory; the scattering intensity increases with decreasing wavelength; thus, its contribution to the long wavelength absorption peak is noticeably less than to the short wavelength peak.

In addition to chlorosomal Bchl, GSB cells may contain Bchl *a*; inside light-harvesting antenna complexes (chlorosomes), per 1000–2000 Bchl *c, d*, or *e* molecules in one photosynthetic unit, there are 100 Bchl *a* molecules inside the FMO (Fenna–Matthews–Olson) protein and in the reaction center [11]. The absorption band of Bchl *a* is at 805–810 nm [12], i.e., outside the long wavelength absorption band of Bchl *d* and/or *e* (650–800 nm), whereas the concentration of Bchl *a* in a water sample with GSB in much lower than the concentration of Bchl *d* or *e*.

3. ABSORPTION SPECTRA OF BACTERIOCHLOROPHYLLS IN EXTRACTS

Figure 2 presents examples of absorption spectra of extracts prepared from water samples with green- and brown-colored GSB. In the absorption spectra of Bchl extracts, as well as in the absorption spectra of GSB cells, there are two main absorption bands. For the sample extract from Lake Trekhtsvetnoe containing Bchl *d* of green-colored GSB, the maxima of these bands are in the ranges of 427–432 nm and 654–658 nm. For the sample extract from the lagoon on Cape Zeleny containing Bchl *e* of brown-colored GSB, the long wavelength band has a maximum in the range of 652–656 nm and the maximum of the short wavelength band his in the range of 461–468 nm. The relatively large value of optical density

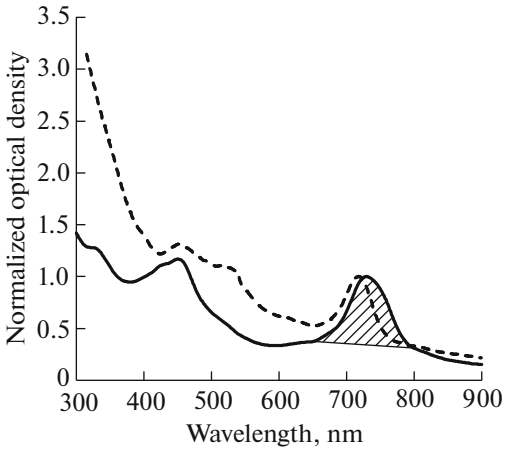

Fig. 1. The absorption spectra of water samples with microorganisms, normalized to the maximum of the long wavelength absorption band of Bchl: (solid line) lake Trekhtsvetnoe, from depth 2.075 m, September 2019, (dotted line) the lagoon on cape Zeleny, from depth 5 m, September 2019. The shaded area corresponds to the long wave absorption band Bchl.

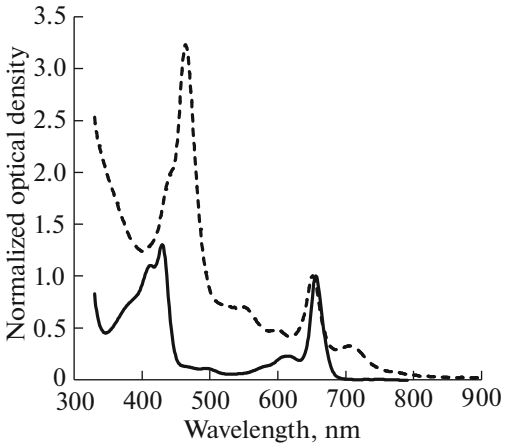

Fig. 2. The absorption spectra of acetone-ethanol extracts of Bchl prepared from water of (solid line) lake Trekhtsvetnoe, from a depth of 2.075 m, September 2019; (dotted line) lagoon on the Cape Zeleny, from a depth of 5 m, September 2019. The spectra are normalized to the maximum of the long wavelength absorption of Bchl.

for the latter band is due to the high content of isorenieratene carotenoid in the cells of brown-colored GSB, the absorption band of which overlaps with the short wavelength absorption band of Bchl *e*.

In acetone–ethanol extract, under the influence of the solvent on GSB cells the destruction of the structure of chlorosome containing highly aggregated pigments of Bchl occurs. After extraction, the maximum absorption of Bchl is shifted to a shorter wavelength region by about 70 nm, which indicates the transition of Bchl from an aggregated form to a monomeric one.

We note that the long wavelength peak of absorption of chlorosomal Bchl with a maximum at 655 nm overlaps with the absorption peak of Chl at 663 nm in acetone, so in extracts with the simultaneous presence of chlorosomal Bchl and Chl, their quantitative determination is not a simple task. On the contrary, the absorption bands of both types of pigments in water are well separated spectrally. The maximum absorption of Chl in algae or cyanobacteria cells is in the range of 670–680 nm [2], whereas the long wavelength peak of Bchl absorption in GSB cells is in the range of 715–735 nm.

The absorption bands of Bchl *a* and chlorosomal Bchl in extracts are not overlapped: in the spectra of acetone extracts, the maximum of long wavelength absorption of Bchl *d* is at 654 nm [13], Bchl *e* at 649 nm [14], and Bchl *a* at 770 nm [15]. Therefore, it can be assumed that the presence of Bchl *a* practically does not affect the determination of chlorosomal Bchl in extracts.

4. DETERMINATION OF BCHL CONCENTRATION FROM THE ABSORPTION SPECTRA OF EXTRACTS

To determine the concentrations of Bchl *d* and Bchl *e*, we used the following formulas derived from the Beer–Lambert–Bouguer law and the definition of optical density:

$$C(\text{Bchl } d) = \frac{D_{655}}{\varepsilon_{\text{Bchl } d} d} \frac{v}{V} \frac{1}{\theta} \times 10^6,$$

$$C(\text{Bchl } e) = \frac{D_{655}}{\varepsilon_{\text{Bchl } e} d} \frac{v}{V} \frac{1}{\theta} \times 10^6,$$

where D_{655} is the optical density of the acetone-ethanol (7 : 2) extract at a wavelength of 655 nm after correction for the light scattering; $\varepsilon_{\text{Bchl } d}$ is the extinction coefficient of Bchl d = 98.0 mg (ml × cm)$^{-1}$ [13]; $\varepsilon_{\text{Bchl } e}$ is the extinction coefficient of Bchl e = 49.6 mg (ml × cm)$^{-1}$ [14]; d is length of the optical path in the cuvette (cm); v is the volume of acetone-methanol extract (ml); V is volume of water in the extract (or filtered water) (ml); θ is the percentage of the filter used for preparing the extract ($\theta = 1$, if there was no filtration).

Correction for scattering was performed by subtracting the minimal optical density in the range from 700 to 850 nm, which was assumed to be due to scattering, from the optical density at 655 nm.

The presence of both types of GSB, green- and brown-colored, in the water basin complicates the determination of the concentration of a single pigment (Bchl *d* or Bchl *e*). In this case, the long wavelength absorption bands of both pigments overlap making a separate determination of the concentration

of either Bchl *d* or Bchl *e* using the spectrometric formulas obtained from the Beer–Lambert–Bouguer law become impossible. In this case, the ratio of the contents of Bchl *d* and Bchl *e* can be determined either using the non-overlapping short wavelength absorption peaks of these pigments in the spectra of extracts (440 nm for Bchl *d* and 470 nm for Bchl *e*), or by using fluorescence analysis. This requires a separate spectrometric study, which is beyond the scope of this paper. Therefore, for Lake Bolshie Khruslomeny and Lake Elovoe, the estimates of the ratio of green- and brown-colored GSB were taken from previous studies, according to which the relative content is 73% of green and 27% of brown GSB for Lake Bolshie Khruslomeny [16] and, correspondingly, 60% and 40% for Lake Elovoe [17]. Taking these ratios into account, the formulas for calculating the concentrations of Bchl *e*, Bchl *d* and for the total concentration can be written in the following form:

$$C(\text{Bchl } d) = \varphi_{\text{Bchl } d}\frac{D_{655}}{\varepsilon_{\text{Bchl } d}d}\frac{v}{V}\frac{1}{\theta} \times 10^6,$$

$$C(\text{Bchl } e) = \varphi_{\text{Bchl } e}\frac{D_{655}}{\varepsilon_{\text{Bchl } e}d}\frac{v}{V}\frac{1}{\theta} \times 10^6,$$

$$C(\text{Bchl } d,e) = D_{655}\left(\frac{\varphi_{\text{Bchl } d}}{\varepsilon_{\text{Bchl } d}} + \frac{\varphi_{\text{Bchl } e}}{\varepsilon_{\text{Bchl } e}}\right) \times \frac{v}{Vd}\frac{1}{\theta} \times 10^6,$$

where $\varphi_{\text{Bchl } d}$ and $\varphi_{\text{Bchl } e}$ are the fractions of Bchl *d* and Bchl *e* in the mixture for a particular water reservoir. This approach for GSB was used for the first time. Below, we will use the following notation: $\left(\frac{\varphi_{\text{Bchl } d}}{\varepsilon_{\text{Bchl } d}} + \frac{\varphi_{\text{Bchl } e}}{\varepsilon_{\text{Bchl } e}}\right) = \varepsilon_{\text{eff}}$, the effective extinction coefficient for a given reservoir.

5. DESCRIPTION OF A NEW METHOD FOR DETERMINING THE CONCENTRATIONS OF BACTERIOCHLOROPHYLLS IN NATURAL WATER SAMPLES

The method proposed in 2018 [18] does not require a number of traditional operations, since the concentrations of Bchl *d* and *e* are determined by the area of the long wavelength absorption band in the absorption spectra of bacterial cells in water. As the first approximation, the spectral range from 650 to 800 nm was chosen and the baseline under the absorption peak was approximated by a trapezoid. The shaded area in Fig. 1 corresponds to the area of the long wavelength absorption band of Bchl in bacterial cells in this water sample and baseline is due to light scattering. Attempts to approximate the baseline by other functions (inverse powers of the wavelength with different parameters) did not increase the accuracy of calculating the area of the long wavelength absorption band of Bchl. Therefore, the linear approximation was chosen as the simplest and most convenient. Light scattering can be taken into account in other ways. As an example, as shown in [8] for GSB, scattering can be reduced by measuring the absorption spectra of GSB cultures in glycerol solutions. However, this method requires additional sample preparation and is not considered in this work. As well, as was shown in [19] for Chl *a*, the true absorption spectrum (without the contribution from scattering) can be obtained using the method of an integrating sphere. In this work, this method is also not considered as unsuitable for subsequent application in a submerged device. Light absorption by dissolved organic matter, which is always present in lake water samples, is negligible in the spectral range of the long wavelength absorption band of Bchl (650–800 nm) and does not significantly affect the measurement of pigment concentration [20, 21].

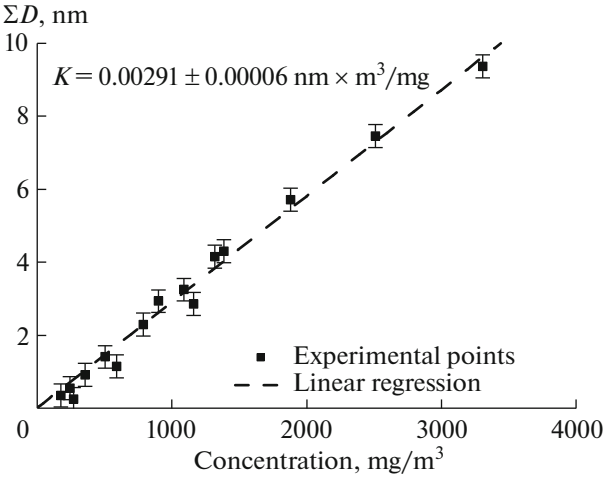

Fig. 3. The correlation between ΣD, the area of the long wavelength absorption band of Bchl in water, and the concentration of Bchl in extracts calculated using the formula obtained from the Bouger law. The data are for lake Trekhtsvetnoe (multi-syringe sampling with 2.5 cm depth increments, March 2018).

For all the studied series of samples, we found a proportional relationship between the value of ΣD (the area of the long wavelength band in the absorption spectra of Bchl in water samples after subtracting the area of the baseline due to scattering) and the concentrations of Bchl, which was calculated using the given above formulas obtained from the Beer–Lambert–Bouguer law. An example of the obtained dependencies is shown in Fig. 3. For each sample series, linear regression coefficients K [nm × m^3/mg] were obtained for proportional dependencies ΣD on the concentration of Bchl calculated using the pho-

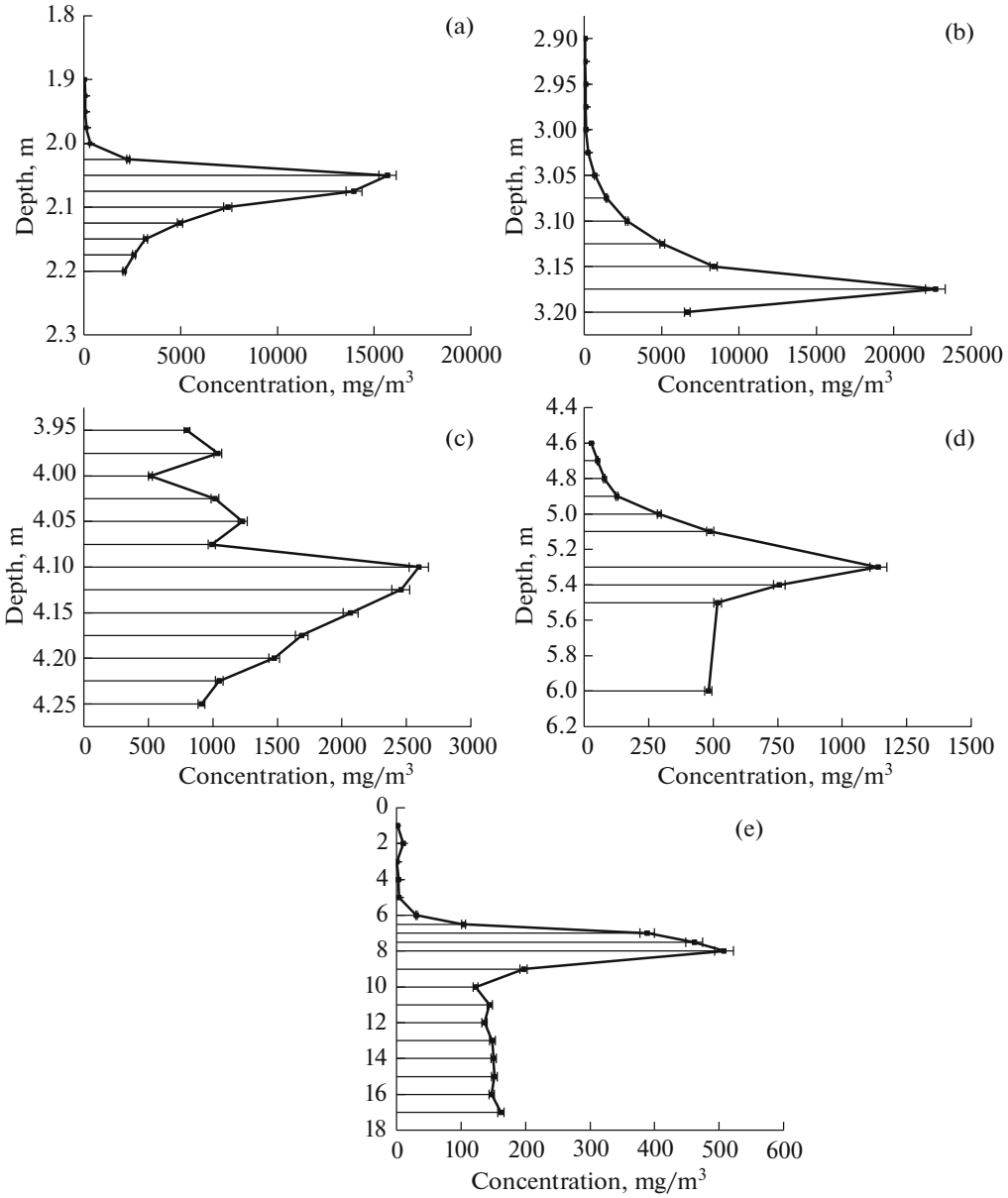

Fig. 4. The depth distribution of Bchl concentration in water basins: (a) Lake Trekhtsvetnoe (sampling by multi-syringe sampler, September 2019); (b) Lake Elovoe (sampling by multi-syringe sampler, September 2019); (c) Lake Bolshie Khruslomeny (sampling with a multi-syringe sampler, September 2019); (d) lagoon on the Cape Zeleny (sampling with a submersible pump, September 2019); (e) Lake Mogilnoye (submersible pump sampling, June 2019).

tometric formulas for extracts. Introducing coefficient A [mg/(nm $\times$ m^3)], which is the inverse of the coefficient K, the formula for calculating Bchl concentration from absorption spectra in water can be written as follows:

$$C(\text{Bchl } e,d)\ [\text{mg/m}^3] = A\ [\text{mg/(nm} \times \text{m}^3)] \cdot \Sigma\text{D [nm]}.$$

For all the studied series of samples, the obtained values of coefficient A were quite close: 344 ± 7 mg/(nm $\times$ m^3) (the green form of GSB with Bchl d, Lake Trekhtsvetnoe, multi-syringe sampling, March 2018), 339 ± 24 mg/(nm $\times$ m^3) (the green culture with Bchl e, strain ZM-2014), 334 ± 8 mg/(nm $\times$ m^3) (mixed community of green and green forms of GSB, Lake Bolshie Khruslomeny, sampling by multi-syringe sampler, September 2018). Therefore, for the final empirical formula, the average value of the coefficient A for the nine studied series was chosen: $A = 336 \pm 9$ mg/(nm $\times$ m^3). Thus, we obtained a formula for calculating the concentration of Bchl from the absorption spectra of microorganisms directly in water:

$$C(\text{Bchl } e,d)\ [\text{mg/m}^3] = 336\ [\text{mg/(nm} \times \text{m}^3)] \cdot \Sigma\text{D [nm]}.$$

This empirical formula was further used to calculate the concentrations of Bchl *d* and *e* in the studied series of water samples from the lakes of Trekhtsvetnoe, Elovoe and Bolshie Khruslomeny, as well as lagoon on Cape Zeleny, taken in September 2019. The good agreement of the data obtained by different methods follows from the correlation of Bchl concentrations determined from the absorption spectra of extracts and water samples with GSB. The spread of correlation coefficients for different series of samples is within the range of 0.9866–0.9991.

To test the method, the distribution profiles of Bchl concentrations for each series of samples were obtained by measuring the absorption spectra of bacteria directly in water (see Fig. 4). Figure 4 shows examples of Bchl concentration distribution over the depth in several meromictic water reservoirs of the White Sea region in September 2019 and Lake Mogilnoye (Barents Sea) in June 2019. These distributions indicate that the depth with the maximum concentration of Bchl in Lake Trekhtsvetnoe is 2.05 m, Lake Elovoe is 3.175 m, Lake Bolshie Khruslomeny is 4.1 m, the lagoon on the Cape Zeleny is 5.3 m and Lake Mogilnoye is 8 m. These data are consistent with the results of measurements in the same reservoirs in previous years, with the concentration of Bchl and the depth of the horizont with its maximum concentration being depended on the season and change in different years. As an example, for Lake Trekhtsvetnoe, the maximum concentration of Bchl *d* was observed at depths of 1.8 m (July–August 2013), 2.0 m (February 2015), 1.825 m (July 2016), 1.85 m (March 2017), 1.9 m (July 2017), 2.1 m (March 2018), 2.05 m (September 2018), 2.175 m (March 2019) [22–24]. Seasonal variations in the concentration and depth of the maximum concentration of Bchl generally depend on hydrological and climatic conditions and illumination in the upper layers of the reservoir.

CONCLUSIONS

Thus, the developed method makes it possible to determine the depth distribution of bacteriochlorophyll of GSB in meromictic reservoirs without extracting pigments and to significantly speed up and simplify the process of determining Bchl concentrations and obtaining the vertical profiles of Bchl distribution over the depth; it can potentially be used to determine Bchl concentrations directly in the water reservoir without sampling using a submerged spectrophotometer.

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