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Fluorescence of chlorosomal bacteriochlorophylls extracted by organic solvents applied for pigment quantification in natural water samples

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ABSTRACT

Environmental monitoring of natural water bodies is important in the Arctic zone for studying their evolution under the influence of climate change and the urbanization processes in the region. In connection with the specific life conditions for anoxygenic phototrophic microorganisms their presence can indicate hydrogen sulfide contamination of the water reservoir and serve as a marker of hydrogen sulfide. This makes the problem of optical diagnostics of phototrophic bacteria very urgent and important. There is a number of spectral methods for determination of chlorophyll-containing microorganisms. The spectral properties of photosynthetic bacterial pigments, bacteriochlorophylls (BChls), are still poorly understood, however. For the first time we applied fluorescence spectra of BChl extracts to receive depth distribution of BChl *d* in the lake. Fluorescence emission spectra were measured using a Solar CM2203 luminescence spectrometer under excitation at wavelength of 425 nm, corresponding to the BChl *d* absorption peak. In September 2020 the maximal concentration of BChl *d* was found at the depth of 2.275 m (16700 mg/m^3) in Lake Trekhtzvetnoye. The thickness of the bacterial plate did not exceed 5 cm, and the pigment distribution was found vertically asymmetric. We emphasize that fluorescence quantification of BChl *d* is more sensitive compared to spectrophotometric one, and it makes possible estimation of ultralow BChl concentrations without water sample pre-concentration.

Keywords: bacteriochlorophyll (BChl), photosynthetic pigments, green sulfur bacteria, White Sea, fluorescence spectra, absorption spectra, depth bacteriochlorophyll distribution.

1. INTRODUCTION

The problem of studying phototrophic microorganisms or their pigments in different layers of water often arises during environmental monitoring of natural water bodies. It is especially important in the Arctic zone for studying the evolution of natural water bodies under the influence of climate change and the urbanization processes in the region. The chemocline zone in the water bodies being on different stages of isolation from the White Sea is typically inhabited by anoxygenic phototrophs[1-3], and the green sulfur bacteria (GSB) are prevailing among them. The GSB are anoxygenic phototrophs that often live in lakes and coastal lagoons with a strong water density stratification in the chemocline region under low sunlight illuminance. Their presence can indicate hydrogen sulfide contamination of the water reservoir. This makes very urgent the problem of optical diagnostics of microorganisms being markers of hydrogen sulfide in natural water.

A number of spectral methods for determination of pigments have been developed for chlorophyll-containing microorganisms[4-6]. Spectrophotometry is the classical method of determining the quantity of chlorophyll in the plankton cells in natural water. It includes collecting a fairly large sample of water, filtering the sample to concentrate chlorophyll-containing organisms, mechanically disrupting the collected cells, and extracting chlorophyll from the destroyed cells into an organic solvent, acetone. The extract is then analyzed by either a spectrophotometric method (absorbance or fluorescence), using the known optical properties of chlorophyll, or by HPLC.

However, the spectral properties of photosynthetic bacterial pigments, bacteriochlorophylls (BChls) are still poorly understood.

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BChls are photosynthetic pigments of GSB, contained in a highly aggregated form in photosynthetic antenna complex, named chlorosome. There are several studies on optical properties of chlorosomal BChls performed on living bacteria[7-8] and their extractions in acetone-methanol mixtures [9-10]. However, to date only absorption spectra of BChls were used for pigments quantification.

This paper presents the study of the fluorescence of the chlorosomal bacteriochlorophyll (BChl *d*) in extracts prepared from natural water from Lake Trekhtzvetnoye containing GSB sampled in September 2020 at the Kandalaksha coast of the White Sea.

2. MATERIALS AND METHODS

2.1 Sampling location for water with phototrophic bacteria

Water samples were taken from different depths from Lake Trekhtzvetnoye, one of the stratified lakes on the Kandalaksha coast of the White Sea (66° 35' 33" N, 32° 58' 43" E). This is a meromictic lake with a fresh water upper 2 meters and salt water near the bottom. At the depth of 2 m the abrupt change of physical and chemical characters appears. This is a boundary between aerobic and sulfide conditions (a redox interface), a strong gradient of salinity and temperature (a halocline and a thermocline, respectively), and the lower limit of sunlight propagation. In this border zone a thin layer of water colored in bright green exists all year round. This layer is a bacterial plate - a liquid bacterial mat with a dense culture of anoxygenic phototrophic green sulfur bacteria [1-2].

Figure 1. Photo of the studied lake Trekhtzvetnoye, September 2020.

The water sampling was performed using a submersible pump from the water surface down to the depth of 7 m, as well as a specially designed multichannel water sampler (23 samples with depths interval of 2.5 cm were taken from the zone of chemocline starting from 2.0 m).

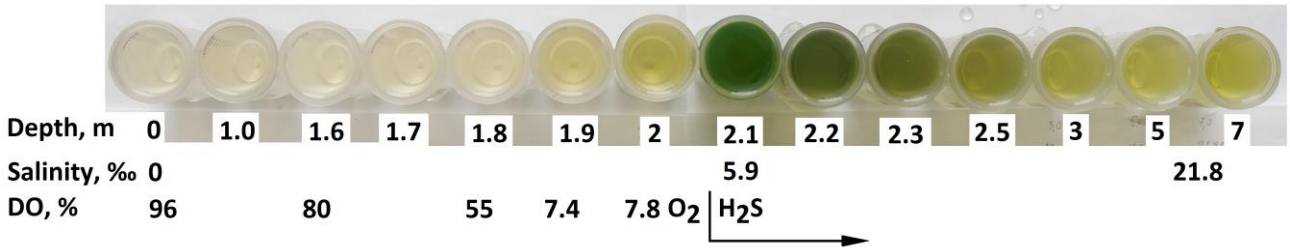

Figure 2. Photo of the water samples taken from various depths from Lake Trekhtzvetnoye, September 2020. DO - dissolved oxygen, saturation in percentage terms. Dissolved oxygen was measured with YSI ProODO optical oxymeter, salinity was measured with YSI Pro conductometer.

2.2 Preparation of pigment extractions and spectral measurements

For the spectroscopic studies extracts in an organic solvent were prepared by adding 1 ml of the water sample with to 4 ml of a mixed solvent acetone-ethanol, taken in a ratio of 7:2.

The absorption spectra were measured on a Solar PB 2201 spectrophotometer in a cuvette with an optical path length of 1 cm. Based on the absorption spectra, the BChl concentration in the extracts was calculated[10-11].

Fluorescence emission spectra were measured using a Solar CM2203 luminescence spectrometer for extracts. After measuring the spectra, the fluorescence intensity was corrected taking into account absorption at both excitation and registration wavelengths by multiplying the detected fluorescence intensity by $10^{0.5 \cdot (Dex + Dem)}$, where Dex and Dem are absorbances at the excitation and registration wavelengths, respectively.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1 Absorption spectra

There are two types of GSB differing in the pigments of the light-harvesting complex, a green-colored and a brown-colored type. The green-colored cultures contain BChl *d* as a main photosynthetic pigment, while the brown-colored cultures contain BChl *e*. Therefore, their absorption spectra demonstrate different bands in extracts of pigments.

The measured absorption spectra of acetone extracts BChl *d* and BChl *e* with different concentration of pigments are shown in Fig. 3. The measured absorbances can be used to determine the BChls concentrations, if the extinction coefficients are known.

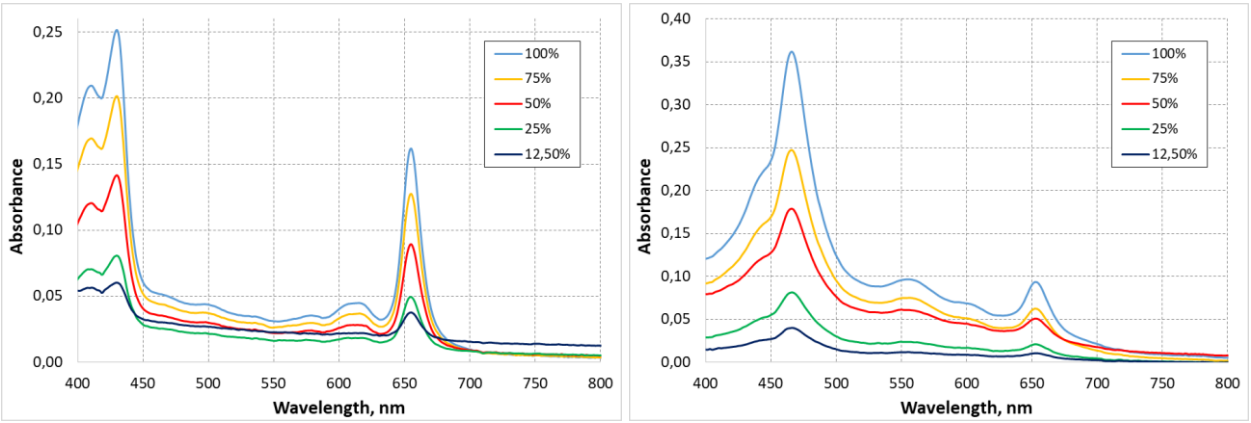

Figure 3. Absorption spectra of acetone extracts of BChl *d* (left) and BChl *e* (right) at various concentrations in acetone.

3.2 Fluorescence of chlorosomal bacteriochlorophylls in organic solvents

The fluorescence emission spectrum of a suspension of living cells of GSB in water has three pronounced bands, one of which has a maximum in the range from 730 to 770 nm, corresponding to the fluorescence of chlorosomal BChl *d* or BChl *e* in a highly aggregated state. The other emission band, with a maximum at 813 nm, corresponds to for the fluorescence of BChl *a*, and the third has a maximum at about 670-675 nm due to the emission of BChl *d/e* in the monomeric form.

In contrast to the BChls in the living cells, the same pigments in organic solvents are presented only in monomeric form, and, therefore, their fluorescence differs in characteristics. Fig. 4 shows fluorescence emission spectra of chlorosomal BChl for extracts of the water samples from Lake Trekhtzvetnoye taken from depths between 2.325 and 2.400 m.

The fluorescence emission spectra of the extracts upon excitation with wavelengths shorter than 620 nm are characterized by two peaks: a main peak with a maximum at about 660 nm and a less intense long-wavelength "shoulder" around 725 nm (Fig. 4).

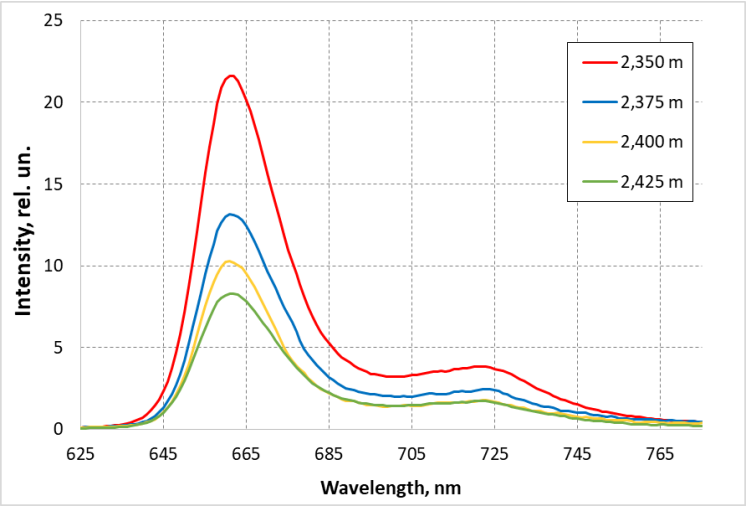

Figure 4. Fluorescence emission spectra of extract of green sulfur bacteria from different depths in Lake Trekhtzvetnoe measured with excitation at 425 nm.

3.3 Quantitative measurements of bacteriochlorophylls from absorption spectra of extractions

To determine the concentration of BChl *d* in extracts scientists use the empirical Overmann-Tilzer formula[12] or its modification based on the Bouguer-Lambert-Beer law applied to absorption spectra of chlorosomal BChl with known extinction coefficients[10]. In its generalized form, the equation for calculating the concentration of the predominant BChl *x* (BChl *a, d,* or *e*) (marked as $C_{BChl\ x}$, µg/L) may be written as follows:

$$C_{BChl\ x} = D/k\ (v/V) \times 10^6,$$

where D is the optical density of the extract in a 1 cm cuvette and *k* is the specific absorption coefficient of the extract at the maximum of the BChl *x* long-wavelength band. It should be noted that *k* depends on BChl type, as well as on the solvent and the wavelength at which optical density is measured[11].

To determine individual BChl *d* or BChl *e* concentrations, the following formulas obtained from the Bouguer-Lambert-Beer law of attenuation of light can used:

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

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

where D_{655} – absorbance value of acetone-ethanol (7:2) extract at a wavelength of 655 nm corrected for scattering

$\varepsilon_{\text{Bchl } d}$ – extinction coefficient of BChl *d* , $\varepsilon_{\text{Bchl } d}$ = 98,0 mg·(ml·cm)$^{-1}$, [13]

$\varepsilon_{\text{Bchl } e}$ – extinction coefficient of BChl *e* , $\varepsilon_{\text{Bchl } e}$ = 49,6 mg·(ml·cm)$^{-1}$, [14]

d – cuvette optical path length (cm)
v – volume of acetone-methanol extract (ml)
V – volume of water in the extract (or filtered water) (ml)
For the quantitative determination of pigments in a water sample, one can use the measurement of the fluorescence of the pigment extract in an organic solvent, and the calibration of the fluorescence intensity against the pigment concentration can be carried out using the absorption spectra by the previously developed method using the extinction coefficient.

Fluorescence measurement is a more sensitive method in comparison with spectrophotometric measurements, that is, it is possible to measure an order of magnitude lower pigment concentrations than the classical method by the absorption spectra of BChl in the extract. In addition, fluorescence measurements are less affected by light scattering, which is inevitable in optical measurements without preliminary removal of cell particles (sedimentation or filtration). However, in natural water bodies in the anoxic zone with sufficient illumination various anoxygenic phototrophic bacteria can be found, therefore several types of BChl can be simultaneously present in extracts. For this reason in the mixture of pigments their emission bands are superimposed. In the future it is planned to develop a multiwavelength fluorescence method (a sequential measurement of emission spectra with several excitation wavelengths) for the simultaneous determination of several bacteriochlorophylls.

3.4 Vertical distribution of BChl *d* from absorption and fluorescence measurements performed on extractions

For the quantitative determination of BChl by fluorescence measurements water samples from Lake Trekhtzvetnoye were taken in September 2020. The sampling from various depths was carried out with a multi-syringe sampler with an interval of 2.5 cm, starting from a depth of 2 m. BChl extracts were prepared from natural water samples by adding 1 ml of water to 4 ml of a mixed solvent acetone-methanol taken in a proportion of 7:2.

It is known that only green-colored forms of green sulfur bacteria containing BChl *d* are observed in significant quantities in the chemocline of Lake Trekhtzvetnoe. This facilitated the task of quantitative determination of the BChl concentration from absorption spectra. However, the pigment concentration could be reliably determined from the absorption spectra for the relatively high pigment concentration. In the case of samples taken in September 2020 only two syringes (numbers 12 and 13 from depths of 2.275 and 2.300 m, respectively) with maximal concentration of the pigment can provide good absorption data for BChl quantification, see Fig. 5 (left). In the remaining samples the light scattering signal interfered, since the removal of cell particles was not carried out.

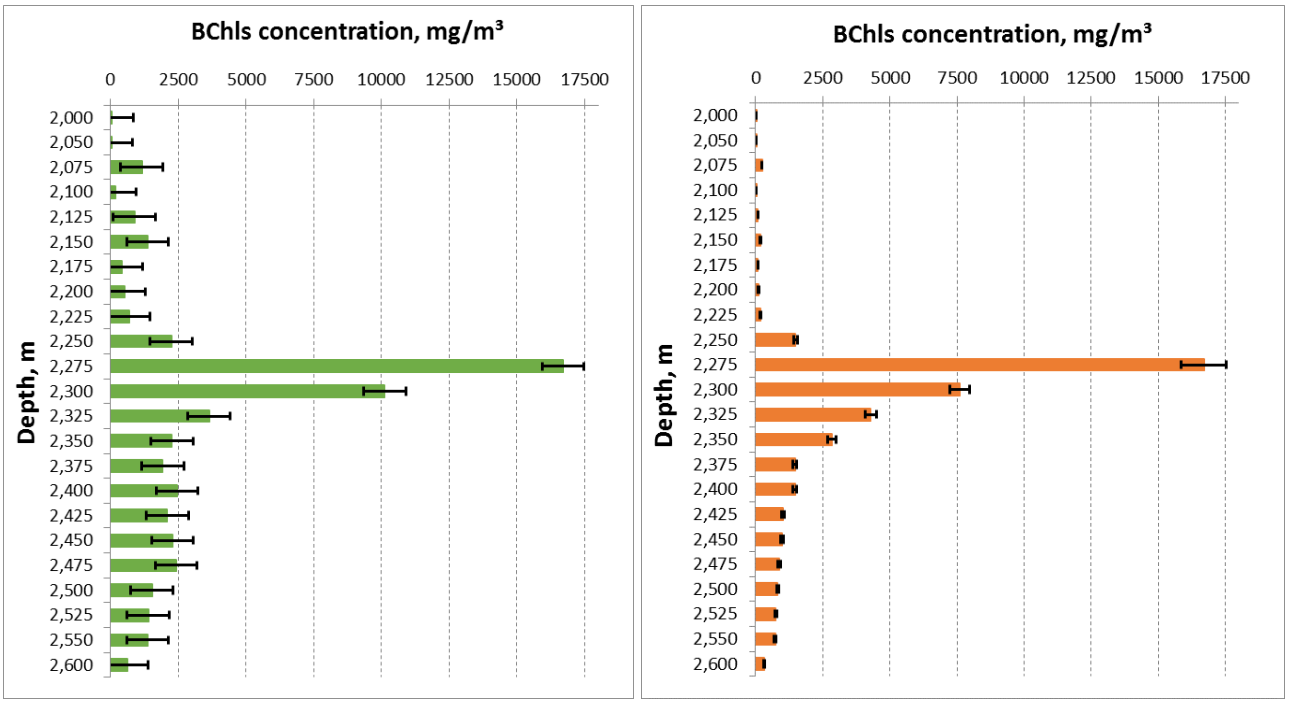

Figure 5. Depth distribution of BChl pigments derived from absorption spectra (left) and fluorescence spectra (right) for extractions prepared for the water samples taken with depths interval 2.5 cm starting from 2.0 m.

Fluorescence emission spectra were measured using a Solar CM2203 luminescence spectrometer for extracts under excitation with light with a wavelength of 425 nm corresponding to the maximum of the absorption band of BChl *d*. The calculation of the concentration of BChl *d* from the absorption spectra was carried out according to the above given formula. The concentration obtained for the sample 12 from a depth of 2.275 was used to determine the conversion

factor connecting the fluorescence intensity and the BChl *d* concentration. The obtained depth distribution of BChl *d* in Lake Trekhtzvetnoye in September 2020 is shown in Fig. 5 (right).

The maximal concentration of BChl *d* was found within chemocline zone at the depth of 2.275 m and was equal to 16700 mg/m^3. The thickness of the bacterial plate (the interval between depths with concentration two times smaller the maximal one) did not exceed 5 cm. The pigment distribution was found vertically asymmetric: it dropped sharply to almost zero above the layer with maximal pigment concentration,and spread smoothly below chemocline to some non-zero value towards the lake bottom.

We emphasize that fluorescence measurement is a more sensitive method in comparison with spectrophotometric measurements, that is, it is possible to measure an order of magnitude lower pigment concentrations than the classical method by the absorption spectra of bacteriochlorophyll in the extract. Note that 1 ml of each sample was used to prepare the extracts; this amount would not be enough to concentrate the sample with the traditional method of measuring the pigment concentration by absorption spectra.

4. CONCLUSIONS

In this work measurements were carried out with extracts of green sulfur bacteria from natural water sampled in September 2020 from different horizons of Lake Trekhtzvetnoye (the coast of the Kandalaksha Bay of the White Sea). As shown by spectral measurements all extracts contain BChl *d*, and in addition to it, BChl *c* was detected in samples at a depth of 2.225 and 2.250 m. The fluorescence intensity at an excitation wavelength of 460 nm is extremely low in the extracts. This fact means that BChl *e*, a typical pigment of brown-colored forms of green sulfur bacteria, is present in a negligible concentration in September 2020 at all horizons of the lake. Only green-colored forms of sulfur bacteria were observed. For the first time we applied fluorescence spectra of extracts of chlorosomal BChls from bacterial cells to receive depth distribution of BChl *d* in Lake Trekhtzvetnoye in September 2020. The maximal concentration of BChl *d* was found at the depth of 2.275 m (16700 mg/m^3). The pigment distribution was asymmetric: it drops sharply to almost zero above the layer with maximal concentration, and spreads smoothly to some non-zero value at a maximum depth about 6 m. The thickness of bacterial plate (the interval between depths with concentration two times smaller the maximal one) did not exceed 5 cm.

Fluorescence measurement is a more sensitive method in comparison with spectrophotometric measurements, that is, it is possible to measure an order of magnitude lower pigment concentrations than the classical method by the absorption spectra of bacteriochlorophyll in the extract.

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