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Fluorescence of chlorosomal bacteriochlorophylls in organic solvents

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Chlorosomal bacteriochlorophylls are the main photosynthetic pigments of green sulfur bacteria, that are anoxygenic phototrophic microorganisms. The spectral properties of chlorophylls of vascular plants, algae and cyanobacteria are well studied, however, the spectral-luminescent properties of their related compounds, bacteriochlorophylls, which participate in anoxygenic photosynthesis, are practically not described in the scientific literature. The polarity of the solvent and the environment have a significant effect on the emission spectra (bacterio)chlorophylls, which is expressed in the spectral shift of the absorption and fluorescence maxima, as well as changes in the fluorescence intensity. The spectral characteristics of bacteriochlorophylls *d* and *e* were obtained in organic solvents such as acetone, methanol, ethanol and isopropanol, as well as in acetone-ethanol (7 : 2) and acetone-methanol (7 : 2) mixtures. These solvents are most often used for the extraction of bacteriochlorophylls from bacterial cells, so the work will be useful for the development of methods for the quantitative determination of chlorosomal bacteriochlorophylls in bacterial cells or in samples of natural water.

Keywords: Bacteriochlorophyll (BChl), fluorescence, absorption spectra, photosynthetic pigments.

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1. Introduction

Spectral and luminescent properties of bacteriochlorophylls (BChls), pigments of phototrophic bacteria, in contrast to related compounds, i.e. chlorophylls (Chl), are almost undescribed in the research literature. Bacteriochlorophylls are a group of photosynthetic tetrapyrrole pigments that are synthesized by various anoxygenic phototrophic bacteria that perform photosynthesis without oxygen release (anoxygenic photosynthesis). In green sulfur bacteria (GSB), the main photosynthetic pigments are BChls *d* and *e*, which are classified as *chlorosome chlorophylls* [1] or, as they used to be called, *chlorobium chlorophylls* [2]. Molecules of BChls *d* and *e*, like those of Chls, have a chlorine macrocyclic ring, the degree of unsaturation of which determines the characteristic spectral features of these compounds [3]. Unlike all other Chls and BChls, BChls *d* and *e* lack the $-COOCH_3$ substituent and have the $-CHOH-CH_3$, which allows molecules to aggregate into certain macromolecular structures and ensures dense packing of BChl molecules in chlorosomes, the light-harvesting GSB complexes [4]. One GSB photosynthetic unit (chlorosome) can contain from 900 to 4500 molecules of BChl *d* or *e* and about 80–250 molecules of BChl *a*. For comparison, a photosynthetic unit of green filamentous sulfur bacteria contains 100–200 molecules of BChl *c* in the chlorosome and about 10 molecules of BChl *a* [5,6].

The absorption spectra of BChl *d* and *e* both in samples of natural water containing microorganisms and in extracts in organic solvents are used to estimate the concentration of GSB in water bodies [7–9]. Methods based on the use of the absorption characteristics of pigments are the most convenient for quantitative estimation. The BChl concentration in a series of natural water samples can be estimated on the basis of the fluorescence spectra of their extracts [9–11] due to the linear dependence of the pigment concentration on its fluorescence intensity. However, the calibration of this method requires knowledge of the pigment concentration and its fluorescence in one of the samples under study. In addition, it is possible to estimate the partial concentrations of two GSB species on the basis of fluorescence spectra of natural water samples containing microorganisms by decomposing the fluorescence maximum into three Gaussian curves with fixed parameters [12]. However, this method does not allow for estimating the exact content of pigments in samples, in contrast to absorption spectroscopy methods. Thus, this line of research is extremely important for monitoring stratified water bodies with sulfide anoxia, which can occur naturally or due to anthropogenic pollution, including relict water bodies in the Arctic region. A common feature of such water bodies is the stable stratification of the water column and the formation of habitats for anoxygenic phototrophic microorganisms (mainly GSB) at the interface between the oxygen and hydrogen sulfide layers.

In biochemical studies, for the isolation of chlorophylls, carotenoids and other lipophilic pigments of bacterial cells, including in the method of high performance liquid

chromatography, the extraction procedure is widely used with acetone, methanol, isopropanol, ethanol and the most commonly used acetone-methanol solution in a volume ratio of 7 : 2 [13–17]. The efficiency of extraction of lipophilic pigments, including BChls, depends on several factors at once: solvent polarity [18], species diversity of organisms, location and orientation of the intracellular pigment, interaction between the solvent and the membrane [19]. In this context, currently it fails to distinguish any one solvent that is completely suitable for the extraction of photosynthetic cell pigments. We have not found to find any studies with results of investigation of the direct effect of organic solvents on the isolation of chlorosomal BChls (BChl *d* and *e*) from cells. For this reason, the results obtained in this study are fundamentally new and practically significant.

2. Subjects of the study and methods of investigation

The subjects of the study were water samples with two species of green sulfur bacteria (*Chlorobiaceae*) — green-colored GSB (the cells of which contain BChl *d* and the carotenoid chlorobactin) and brown-colored GSB (containing BChl *e*e photosynthetic pigment and carotenoid isorenieratin) [1]. Natural water samples were taken during the expedition in September 2022 by a submersible pump from the chemocline of coastal stratified lakes, separated from the White Sea in the Kandalaksha Bay. In the chemocline of these lakes, only one GSB species was present, which made it possible to use water samples from them as sources of certain BChls. The water with green-colored GSB was sampled from the Trekhtzvetnoe lake from a depth of 2.2 m, and the water with brown-colored GSB was taken from the lagoon at the Zeleny cape from a depth of 5.3 m. The choice of depth was determined by the location of the zone with the maximum concentration of microorganisms in the water column. The purity of the pigment contained in natural water samples was tested using their fluorescence emission spectra, which have different parameters (wavelength at the peak and fluorescence band width) depending on the presence of one or another type of BChls [12]. Microorganisms from the lakes were concentrated on glass fiber filters with a pore size of 2 μm using a standard filtration unit. After the filters were dried, concentrated extracts were prepared from the obtained samples.

2.1. Preparation of extracts

This study used four organic solvents (acetone, methanol, ethanol and isopropanol) and two prepared mixtures: acetone-ethanol solution (7 : 2) and acetone-methanol solution (7 : 2). In order to avoid the influence of the solvent on the process of pigment release from bacterial cells into the solution, two concentrated extracts of BChl *d* and *e* were preliminarily prepared in acetone, the same volume of which was subsequently diluted 60 times with each of the above-mentioned solvents. The solutions resulted from this dilution contained the same amount of BChl *d* or *e* in the monomeric form. The influence of acetone on the other solvent used in such solutions was neglected.

2.2. Method for determination of BChl concentration in extracts

According to the Bouguer–Lambert–Beer law, monochromatic light with a certain wavelength, when passing through an optically thin layer of a substance, is attenuated in proportion to the thickness of the thin layer and the concentration of the substance absorbing the light. In a layer of finite thickness, the attenuation will take place in accordance with an exponential law. Optical density D, which is defined as the decimal logarithm of the ratio between the intensity of incident light of the sample and the intensity of light outgoing from the sample, is proportional to the concentration of absorbing molecules. For a BChl extract with a concentration C [mol/l] and an optical path length l [cm] the following can be written:

$$D = \varepsilon_{10} C l,$$

where ε_{10} is a proportionality factor, called the decimal molar extinction coefficient [l/(mol·cm)]. The decimal molar extinction coefficient is related to the molar extinction coefficient as follows: $\varepsilon_{10} \sim 0.4343\varepsilon_{\text{mol}}$. Depending on the dimension of the concentration C (expressed in g/l or mol/l), specific ε and molar ε_{mol} extinction coefficients are distinguished, which are related to each other by the following relationship:

$$\varepsilon M = \varepsilon_{\text{mol}},$$

where M is molar mass of the pigment (BChl) [20].

When switching to the specific extinction coefficient ε and the concentration with the dimension of [g/l], the final formula is obtained for calculating the concentration of BChl *d* or *e* in acetone, which has been used in this study:

$$C = D/0.4343\varepsilon l,$$

where D is optical density of the extract at a wavelength of 655 nm, l is optical path length = 1 cm, ε is extinction coefficient in acetone, equal to 98 l/g·cm for BChl *d* [2] and 58.6 l/g·cm for Bhl *e* [13].

2.3. Spectral measurements

The optical density spectra of the samples were measured by a Solar PB2201 spectrophotometer in a wavelength range from 350 to 900 nm. Fluorescence emission spectra were recorded by a Solar CM2203 fluorometer at an excitation wavelength of 425 nm in a wavelength range from 500 to 800 nm (for extracts) and at an excitation wavelength of 440 nm in a wavelength range from 600 to 820 nm (for two samples of natural water). The cell compartment temperature was set to 15°C. A standard quartz cell with a

Table 1. Wavelengths (in nm) corresponding to the optical density peaks for extracts of BChl *d* and *e* (λ_{max}) and values of optical density *D* (in brackets) at the long-wavelength peak

Pigment	Solvent	Formula	λ_{max}, (*D*)
BChl *d*	Acetone	$(CH_3)_2CO$	412, 430, 615, 655 (0.099)
	Acetone-Ethanol (7:2)	–	412, 431, 617, 657 (0.097)
	Acetone-Methanol (7:2)	–	413, 431, 616, 657 (0.083)
	Isopropanol	$(CH_3)_2CH(OH)$	413, 432, 617, 659 (0.094)
	Ethanol	C_2H_5OH	415, 433, 622, 660 (0.087)
	Methanol	CH_3OH	413, 433, 613, 661 (0.082)
BChl *e*	Acetone	$(CH_3)_2CO$	412, 430, 615, 655 (0.059)
	Acetone-Ethanol (7:2)	–	412, 431, 617, 656 (0.052)
	Acetone-Methanol (7:2)	–	413, 431, 616, 657 (0.053)
	Isopropanol	$(CH_3)_2CH(OH)$	413, 432, 618, 657 (0.053)
	Ethanol	C_2H_5OH	414, 434, 612, 659 (0.044)
	Methanol	CH_3OH	415, 433, 615, 661 (0.055)

Table 2. Wavelengths λ_{max} (v nm) corresponding to fluorescence peaks for extracts of BChl *d* and *e* and intensity at the peak I_{fl} (in relative units, in brackets), the values of the dipole moment μ [25] are specified for solvents

Pigment	Solvent	Formula	μ, D	λ_{max} *enen*(I_{fl}
BChl *d*	Acetone	$(CH_3)_2CO$	2.84	657 (96.2), 713 (11.6)
	Acetone-Ethanol (7:2)	–	–	659 (74.2), 716 (10.3)
	Acetone-Methanol (7:2)	–	–	661 (60.4), 717 (9.1)
	Isopropanol	$(CH_3)_2CH(OH)$	1.66	662 (57.1), 718 (8.6)
	Ethanol	C_2H_5OH	1.69	663 (49.8), 719 (8.4)
	Methanol	CH_3OH	1.70	665 (37.7), 720 (7.4)
BChl *e*	Acetone	$(CH_3)_2CO$	2.88	657 (43.2), 712 (5.1)
	Acetone-Ethanol (7:2)	–	–	659 (33.4), 715 (4.6)
	Acetone-Methanol (7:2)	–	–	660 (31.8), 717 (4.7)
	Isopropanol	$(CH_3)_2CH(OH)$	1.66	662 (28.4), 717 (4.3)
	Ethanol	C_2H_5OH	1.69	663 (24.3), 719 (4.1)
	Methanol	CH_3OH	1.70	665 (16.0), 719 (3.1)

tight-fitting lid with an optical path length of 1 cm was used for the spectral measurements. The fluorescence emission spectra were corrected for absorption using the following formula:

$$I_{corr} = I \cdot 10^{0.5(D_{ex}+D_{em})},$$

where I_{corr} is corrected fluorescence intensity value, I is instrumental fluorescence intensity value, D_{ex} and D_{em} are values of the optical density of the sample at the wavelength of excitation and emission, respectively.

3. Experimental results

3.1. Absorption spectra

Bacteriochlorophylls *d* and *e* in bacterial cells are in a highly aggregated state and have two absorption peaks: in the shortwave (440−460 nm, the Soret band) and longwave (730−750 nm) regions [21]. In organic solvents, the absorption maxima are shifted to the short-wavelength region of the spectrum due to the transition of the aggregated state of the BChl pigment to the monomeric form [1]. All spectra of BChl solutions have two clearly distinguished absorption bands in the short and long wavelength regions (Fig. 1). The short-wave band of the green-colored form of GSB (the main photosynthetic pigment of BChl *d*) consists of two overlapping bands with peaks in the region of 412−415 and 430−433 nm (depending on solvent), which corresponds to the absorption of light by BChl *d* and carotenoids, the main of which is chlorobactin [1]. In the long-wavelength region of the spectrum, there is a clearly distinguished absorption peak at 655−661 nm (the wavelength depends on the solvent) next to a less noticeable peak at a wavelength of 613−622 nm, corresponding to the absorption of BChl *d* only [2,22,23]. The brown-colored form of GSB differs from the green-colored form by the enhanced absorption in the blue-green wavelength region of the visible spectrum, which explains the possibility of their habitation in deeper layers of water, where light of this spectral range penetrates [24]. The short-wave band of the brown-colored form of GSB

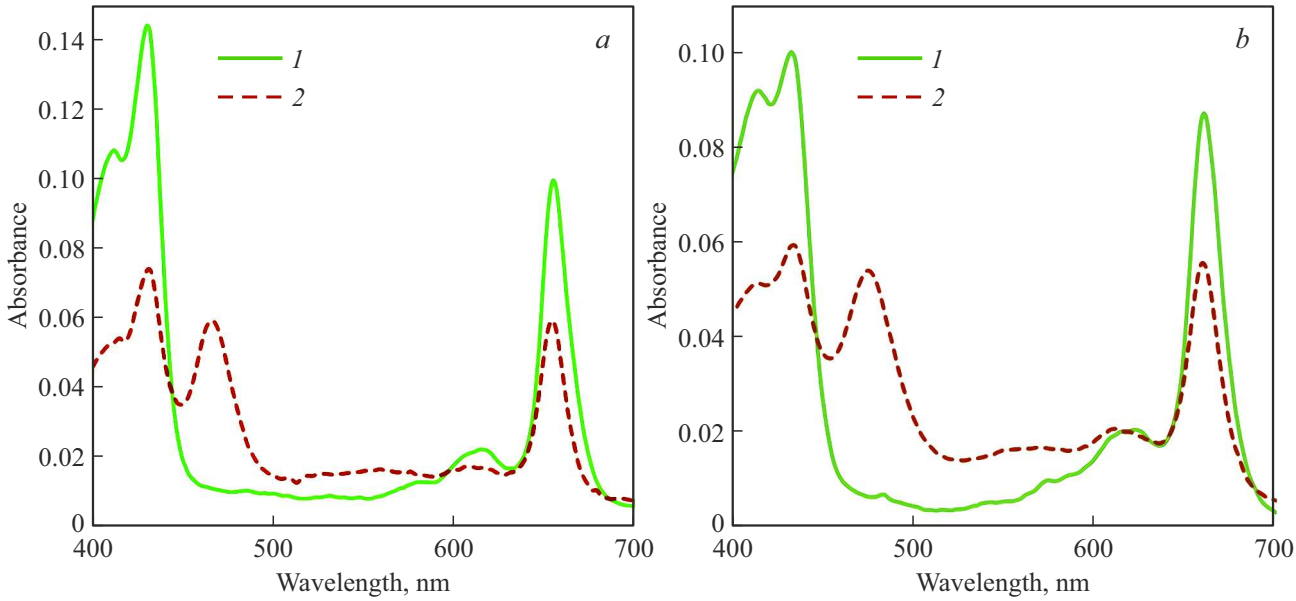

Figure 1. Absorption spectra of BChl *d* (*1*), BChl *e* (*2*) in acetone (*a*) and ethanol (*b*).

(the main photosynthetic pigment of BChl *e*) consists of three overlapping bands with peaks at 412–415 and 430–434 nm (depending on the solvent) corresponding to the absorption of BChl *e* and carotenoids, as well as a clearly distinguished band with a peak at 466–479 nm (Table 1) due to the absorption of a special carotenoid — isorenieratin [23].

Thus, in different solvents, the location of the short-wavelength peaks of BChl *d* and *e* differed by no more than 3 nm, while the main long-wavelength peak of BChl *d* and *e* shifted by 6 nm toward the long-wavelength region of the spectrum in the series of solvents used: acetone → acetone-ethanol (7 : 2) → acetone-methanol (7 : 2) → isopropanol → ethanol → methanol.

Using the optical density values of the acetone extract and the extinction coefficients of BChl *d* and *e* in acetone known from the literature, the concentrations of each pigment in the prepared solutions were calculated (Sec. 2.2). The resultant values were 2.4 mg/l for all solutions containing BChl *d*, and 2.1 mg/l for solutions containing BChl *e*. Despite the similar values of concentrations, the absorbing abilities of the pigments differ greatly, as evidenced by the difference in the optical density peaks of the absorption curves. Thus, the optical density of BChl *d* exceeded the optical density of BChl *e* by almost 2 times in solutions of acetone-ethanol (7:2) and isopropanol. The ratio of peaks in the short-wavelength region was also different and depended on the solvent (Fig. 2).

3.2. Fluorescence spectra

Fluorescence emission spectra were obtained for BChl extracts in various organic solvents at an excitation wavelength of 425 nm (Fig. 3). The excitation wavelength was chosen experimentally based on the peaks of the absorption spectra or fluorescence excitation spectra, where the absorption or emission peak was at a wavelength of 425 nm. All spectra had the main fluorescence peak at wavelengths from 657 to 665 nm and the second peak with a much lower intensity, the maximum of which was at wavelengths from 712 to 720 nm, depending on the solvent (Table 2).

The BChl fluorescence peaks shifted to longer wavelengths in the following solvent series: acetone→acetone-ethanol (7:2)→acetone-methanol (7:2)→ isopropanol→ethanol→methanol, where the fluorescence peaks of BChl *d* or *e* in acetone were at the shortest wavelengths, and peaks of BChl in the methanol solution were in the longest wavelengths. At the same time, the fluorescence intensity for the same series of solvents decreased, despite the same concentration of BChl in the solution. For example, the emission intensity of BChl *d* and *e* in methanol turned out to be more than 2.5 times lower than that in acetone.

In [26], the BChl *d* fluorescence was studied in three different solvents: methanol, hexanol-saturated water and pure water, at the same BChl concentration. It has been shown that the fluorescence intensity of BChl *d* in methanol is much higher than that in hexanol-saturated water. The strong extinction of radiation in the second case was due to the interaction of the fluorophore with the surrounding solvent molecules.

It is worth to note that in this study it was found that the wavelengths of fluorescence peaks of BChl *d* and *e* coincide with each other with an accuracy of 1 nm for both pigments in each of solvents, as well as the shape of the emission curve does (Fig. 4, *a*), despite the different concentrations of the pigment (2.4 mg/l for BChl *d* and 2.1 mg/l for

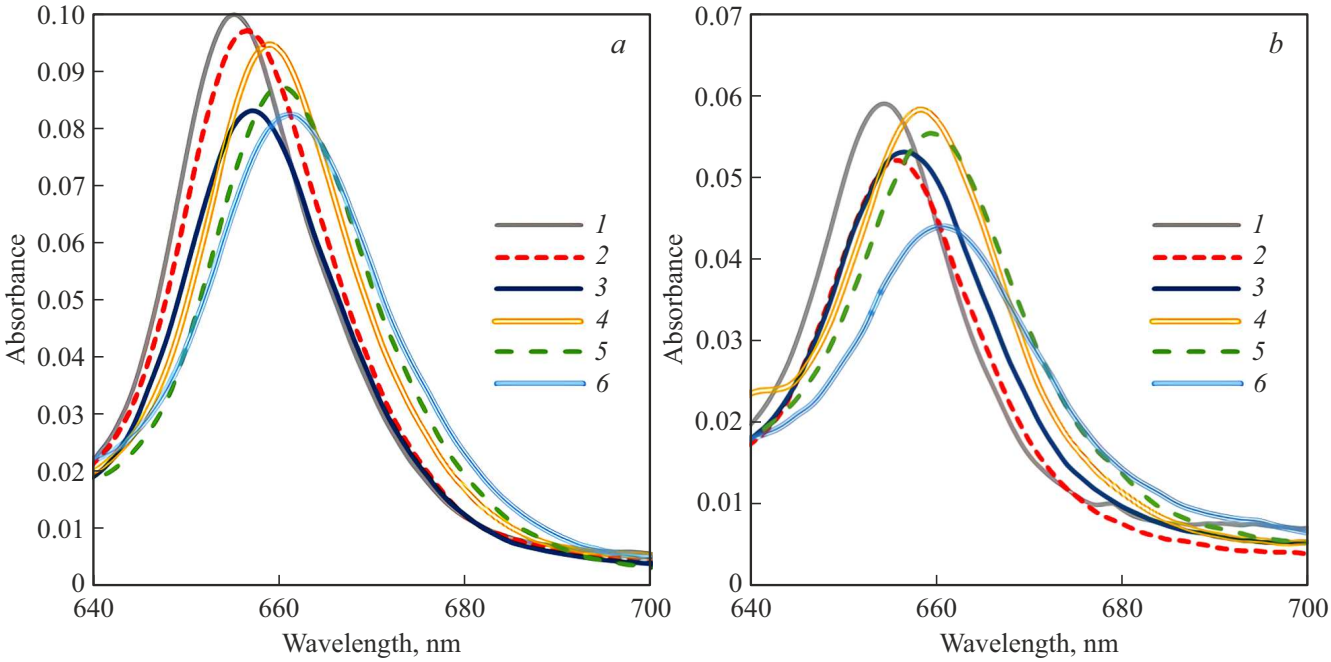

Figure 2. Long-wavelength absorption band of BChl *d* (*a*) and BChl *e* (*b*) solutions in acetone (*1*), acetone-ethanol (7 : 2) (*2*), acetone-methanol (7 : 2) (*3*), isopropanol (*4*), ethanol (*5*), methanol (*6*).

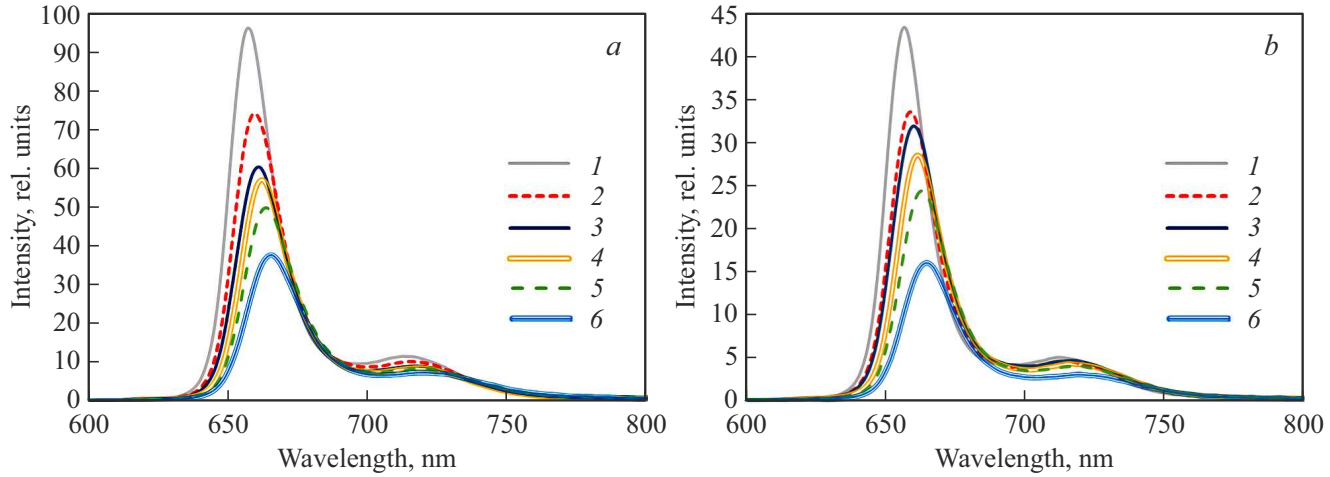

Figure 3. Fluorescence emission spectra of BChl *d* (*a*) and BChl *e* (*b*) in acetone (*1*), acetone-ethanol (7 : 2) (*2*), acetone-methanol (7 : 2) (*3*), isopropanol (*4*), ethanol (*5*), methanol (*6*) (at an excitation wavelength of 425 nm).

BChl *e* and different emissivities. For comparison, the fluorescence spectra were measured for the natural water with phototrophic bacteria from lakes, which was used to prepare extracts. Light with a wavelength of 440 nm was used as the excitation, in the same way as in [9,12,21]. Unlike BChl in extracts, the fluorescence peak wavelengths of BChl *d* and *e* in bacterial cells are separated from each other by 20 nm, which has also been observed in previous studies [12,21]. The fluorescence peak of bacterial cells with BChl *e* (brown-colored form of GSB) is at a wavelength of 745 nm, and in the case of BChl *d* (green-colored form) it is at 765 nm (Fig. 4, *b*).

4. Results and discussion

In an organic solvent, BChl molecules are in a monomeric state; therefore, the change in the absorption and fluorescence spectra is explained by the solvation of molecules and the effect of the solvent on the electron levels of molecules. The solvent affects the electron shell of the BChl *d* or *e* molecule, changing the energy of the electron energy levels

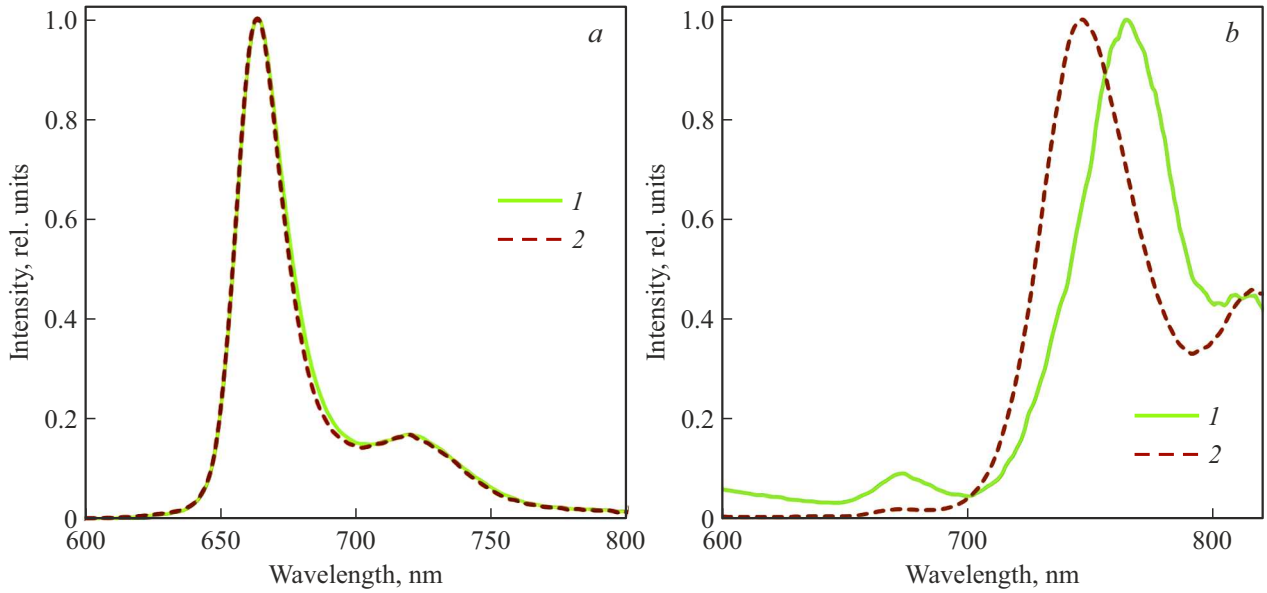

Figure 4. Fluorescence spectra normalized to the long-wavelength fluorescence peak: BChl *d* (*1*), BChl *e* (*2*) in acetone (*a*) and in water (*b*) at excitation wavelengths of 425 and 440 nm respectively.

in the molecule, which affects the position of the bands in the absorption spectra. In this case, the energy level of the ground state decreases by the amount of solvation energy, which depends on the strength and nature of the interaction of porphyrin molecules (which include BChl) with the solvent [27]. It is believed that the absorption spectra are less sensitive to the specific characteristics of the solvent (for example, to polarity) than the fluorescence spectra [25], which is also observed in this study, where the change in the position of the optical density peak is not as large as the change in the position of fluorescence peak in various solvents. In an excited state, the nature of the interaction of porphyrin molecules with the solvent changes due to a change in the state of the electron shell of the molecule due to its polarization by the electric vector of the light wave, i.e. not only the electron state of the emitting fluorophore changes but also the environment surrounding it, which contains solvent molecules oriented around the dipole moment of the excited state. In this case, the polarity of the solvent can have a significant effect on the emission spectra [28].

The shift of the BChl fluorescence peak in various organic solvents can be explained by the difference in the polarity of the solvent molecules, as well as by the different ability to bind with BChl molecules. Typically, the fluorophore has a larger dipole moment in the excited state than in the ground state. After excitation, the dipoles of the solvent can reorient, which lowers the energy of the excited state. As the polarity of the solvent increases, this effect becomes stronger and leads to emission at lower energies or higher wavelengths [25].

One of the indicators of the polarity of a substance at the molecular level is the dipole moment of the molecule; it characterizes the electrical properties of the molecule as a system of charged particles [29]. For the observed sequence of solvents corresponding to the shift of absorption and BChl fluorescence peaks, (acetone→acetone:ethanol (7:2)→acetone:methanol (7:2)→isopropanol→ethanol→methanol) acetone ($\mu = 2.84$ D) has the largest dipole moment, however, other solvents, although they have smaller dipole moments (Table 2, [29]), do not demonstrate an arrangement in descending order. Consequently, the solvent polarity and the environment have a significant effect on the BChl emission spectra; however, it does not seem possible to explain the observed changes only by the influence of the solvent polarity.

BChl *d* and *e* related compounds of microorganisms are chlorophylls (Chl) *a* and *b* of plants. All of them are phytochlorins with one reduced ring in the chemical structure. Bacteriochlorophyll *e* has a -CHO substituent, which is also present in Chl *b*, which leads to the similarity of their spectral characteristics [4]. In solutions, the absorption spectra of BChl *d* are very similar to the absorption spectra of Chl *a*, however, in contrast to the BChl in monomeric state, the difference between wavelengths of absorption peaks of Chl *a* and *b* in acetone is 17 nm, and in methanol it is 13.2 nm [30], i. e. the difference is significant as compared to similar values observed for BChl *d* and *e*.

The fluorescence spectra of Chl *a* and *b*, as well as the fluorescence spectra of BChl *d* and *e*, consist of two bands, one of which corresponds to electronic transitions between singlet energy levels in the fluorophore molecule, and the other (with a lower intensity and located in the longer wavelength region of the spectrum) is associated with vibrational modes [31]. The wavelength of the absorption peak of Chl *a* in acetone is 663 nm, and for Chl *b* it is

645 nm. The main fluorescence peaks of Chl *a* and *b* in acetone are located at wavelengths of 669.5 and 654.6 nm, respectively [30,32]. Thus, the Stokes shift for Chl *a* in acetone is 6.5 nm, and for Chl *b* it is 9.6 nm. In the case of BChl *d* and *e* the Stokes shift varies from 2 to 4 nm depending on the solvent. Smaller values of the Stokes shift are indicative of lower energy losses during its migration, which is an evidence of a higher efficiency of energy transfer in BChl molecules as compared with Chl.

The coincidence of spectral characteristics (wavelengths of fluorescence peaks, shape of emission curves) of BChl *d* and *e* in all organic solvents under study and their significant difference in aqueous solutions indicate a different effect of the aggregation on the spectral properties of molecules. In the highly aggregated state, BChl *d* exhibits fluorescence at longer wavelengths as compared with BChl *e*. At the same time, the spectral properties of BChl *d* and *e* monomers observed in organic solvents coincide in many respects.

5. Conclusion

In the course of the work, the absorption and fluorescence spectra of chlorosomal BChls (BChl *d* and *e*) were measured in in some organic solvents: acetone, methanol, ethanol, and isopropanol, as well as mixtures-solutions of acetone-ethanol (7:2) and solutions of acetone-methanol (7:2). The similarity of absorption wavelengths and emission fluorescence peaks of both types of BChls (*d* and *e*) in the monomeric form is shown. However, the dependence of these spectral characteristics on the solvent was revealed. The sequence of solvents corresponding to the shift of peaks of the absorption and fluorescence spectra to the region of longer wavelengths was determined: acetone→acetone:ethanol (7:2)→acetone:methanol (7:2) →isopropanol→ethanol→methanol. Our observations may be an evidence of different interactions of the fluorophore with solvent molecules.

These solvents are widely used in biochemical studies, as well as in calculating the concentration of BChl in cell cultures or in natural water. The data obtained make it possible to adjust the available spectral methods depending on the use of a particular solvent, as well as to select a suitable medium for the extraction of pigments. The regularities observed in the study are explained from the point of view of the theory of solvatofluorochromia, assumptions are made about the existing features of the absorption and fluorescence of BChl in extracts.

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Conflict of interest

The authors declare that they have no conflict of interest.

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