

SPECTROPHOTOMETRIC QUANTIFICATION OF CHLOROSOMAL BACTERIOCHLOROPHYLL IN INTACT CELLS OF GREEN SULPHUR BACTERIA: MONOCULTURES AND NATURAL WATER

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ABSTRACT

In oceanographic and limnological studies, spectrophotometry combined with extraction of pigments by organic solvents became a routine procedure for chlorophyll (*Chl*) quantification in algae and cyanobacteria from oxic layers. However, for bacteriochlorophyll (*BChl*) of phototrophic bacteria inhabiting the oxic-anoxic interface and deeper anoxic zones, photometric determination of pigment becomes complicated because of overlapping absorption peaks from different chlorophylls, bacteriochlorophylls and phaeopigments in the solvent. The work is aimed to develop a new technique of *BChl* estimation in aquatic environments and to test this method using natural water samples with green sulphur bacteria (GSB, *Chlorobiaceae*). Absorption measurements were carried out using a laboratory spectrophotometer on monocultures of green-coloured as well as brown-coloured GSB in water (19 strains), along with natural water samples from the White Sea region containing either green-coloured or brown-coloured GSB. The far-red absorption peak with a maximum at 720-725 nm attributed to chlorosomal *BChl* in intact cells had practically the same position for all studied cultures. Its peak area (ΣD) was determined for each sample and compared with the total chlorosomal *BChl* content estimated from absorption spectra of extracts prepared from the same specimen. For all samples containing either green-coloured or brown-coloured GSB a good correlation between ΣD and *BChl* concentrations was found, with different regression slopes, however. Differences between the slopes were related to variations on mass-specific absorption coefficients between *BChl*. We provide formulas to evaluate the *BChl* content in intact GSB cells using absorption measurements performed without sample pre-treatment and pigment extraction. The suggested method can be used in communities where one type of microorganisms predominates (either green-coloured or brown-coloured GSB).

KEYWORDS

Absorbance spectra, bacteriochlorophyll, pigment concentration, green sulphur bacteria, stratified lakes, acetone-methanol extracts.

INTRODUCTION

In lakes and coastal lagoons with strong water stratification, the oxic-anoxic interface provides favourable conditions for mass development of specific microorganisms confined to different sublayers of the chemocline, the zone with high chemical gradients (1,2). Eukaryotic algae and cyanobacteria are mostly located in oxic layers, whereas purple sulphur bacteria grow at the oxic-anoxic

interface and below, and green sulphur bacteria (GSB) occur primarily in deeper anoxic layers (3,4).

Spectrophotometric measurements are frequently used in oceanographic and limnological studies for photosynthetic pigment quantification (5,6). Common steps to all protocols based on spectrophotometry include separation of algal (bacterial) cells from water through filtration or centrifugation, extraction of pigments with an organic solvent (usually acetone-based), storage of the extracts in a dark place for several hours or days, centrifugation of the extracts to remove scattering particles, registration of absorption spectra with a laboratory photometer, and finally calculation of the pigment concentration using empirical correlations. For chlorophyll (*Chl*) in algae and cyanobacteria, a routine procedure in despite of several factors affecting the yield of pigment extraction has been established: the solvent to be used, the cell wall disruption technique, the extraction time and the use of different spectrophotometric equations.

Quantification of bacteriochlorophylls (*BChls*) in phototrophic bacteria living at the oxic-anoxic interface and deeper anoxic zones is even more complicated because of the interference of absorption bands of various *Chls*, *BChls* and phaeopigments in extractions and possible degradation of *BChl* pigments exposed to the air during laboratory manipulations. This work is aimed to develop a new technique of *BChl* evaluation in intact bacterial cells directly in aquatic environments without any sample pre-treatment. As a first approach we intend to elaborate the algorithm of *BChl* quantification for bacteria containing mainly one type of chlorosomal *BChl* (*d* or *e*) and to apply this algorithm using a set of monocultures, as well as natural water samples with green sulphur bacteria collected from stratified lakes separating from the White Sea.

OBJECTS AND METHODS

Bacterial monocultures

The green sulphur bacteria (*Chlorobiaceae*) are known as obligate anaerobic photoautotrophic bacteria from the phylum *Chlorobi* (7). These bacteria with anoxygenic type of photosynthesis live in aquatic environments rich in hydrogen sulphide. The GSB use light-harvesting complexes called chlorosomes attached to the cytoplasmic membrane carrying photosynthetic pigments. The type and intracellular pigment content is of high ecological relevance, since GSB are distinguished from other phototrophs by strong adaptation to low light, allowing them to colonize anoxic deep waters or sediments with extremely low light availability. The green-coloured strains of GSB contain *BChl c* or *d* and the carotenoid chlorobactene. The brown-coloured GSB species contain *BChl e* and the carotenoid isorenieratene.

We studied monocultures, the cultures containing only one type of GSB, of green-coloured (9 monocultures) and brown-coloured (10 monocultures) GSB isolated from four lakes being on different stages of separation from the White Sea and located at the Kandalaksha Bay: a lagoon on the cape Zeleny, and the lakes Trekhtzvetnoe, N. Ershovskoe and Kislo-Sladkoye (8).

Water sampling sites: lakes separated from the White Sea

The salt and brackish lakes as well as coastal lagoons formed by gradual isolation from the White Sea resulted from the postglacial isostatic uplift of the sea bottom and shores. They are unique ecosystems with an apparent vertical physicochemical stratification due to meromixis. The interesting phenomenon in such lakes is an appearance of thin coloured layers around the oxic-anoxic interface caused by high abundance of bacterial phototrophs (9,10). Since the water bodies of that type demonstrate stable stratification, the anaerobic phototrophic microorganisms are not disturbed by water circulation. Along the coastline of the Kandalaksha Bay, several lakes are known to be at different stages of their separation from the White Sea (11,12).

The initial stage of isolation from the sea is represented by a saline lagoon on the cape Zeleny characterized by semidiurnal tides of lower amplitude than in the sea. Nevertheless, the lagoon is stratified and consists of three layers with different salinity and two gradient zones between them. At 4-5 m depth the chemocline (redox interface) is situated which could be coloured in red in some seasons by cryptophyte flagellates *Rhodomonas* (in the redox-positive zone) and brown-coloured

sulphur bacteria (below the redox zero) (13). In the stratified salt lagoon on the cape Zeleny the bacterial community of the chemocline is represented by the brown coloured *Chlorobium* strain. The intermediate stage of isolation is represented by two lakes. In lake Kislo-Sladkoe, sea water enters monthly with high syzygy tides. Vertical stratification in this lake is similar to the lagoon on the cape Zeleny. In lake N.Ershovskoye, the freshwater is at the top and with marine salinity at the bottom with maximum depth of about 2.3 m. The green coloured *Chlorobium* is located at 2 m depth, close to the bottom. The lake Trekhtzvetnoe is at the final stage of its isolation from the sea because marine waters do not penetrate the reservoir. Around the chemocline, this lake contains a layer of bright green colour with a specific microbial community. The anoxygenic photosynthetic bacteria are represented there by *Chlorobium phaeovibrioides*.

Monocultures of green sulphur bacteria were isolated from natural water sampled in 2012-2014 in the four lakes described above, and then cultivated at the Winogradsky Institute of Microbiology, Research Center of Biotechnology of the Russian Academy of Sciences (Moscow, Russia).

Water with microorganisms for the optical measurements was sampled from different depths in the lake Trekhtzvetnoe twice, in March and July 2017. In each expedition we used two ways of water sampling: water pumping through plastic tubes submerged at certain depth (depth interval 10 cm, one sample per depth) and the water collection with a multisyringe device (depth interval 2.5 cm, one syringe per depth).

Absorbance measurements

Absorbance spectra of GSB in an aqueous medium (monocultures in growth medium or natural water samples) were registered using the spectrophotometer Solar PV1251 (Belarus) in the spectral range of 400-900 nm, in standard quartz cuvettes with an optical path length of 10 mm, and with distilled water as a baseline standard. To estimate the total content of chlorosomal *BChl* (*d* and *e*), extracts of pigments were prepared using an acetone-methanol mixture (7:2) as a solvent (1 ml of water with GSB was added to 4 ml of the acetone-methanol mixture) and kept in darkness for one day prior to the measurements. The absorbance spectra of extracts were registered with respect to the solvent mixed with distilled water in the same proportion as it was in the extracts.

RESULTS

Absorbance spectra

The absorbance spectra of GSB living cells in water show two main peaks. The far-red *BChl* absorption with a maximum at 720-725 nm is very similar for green- and brown-coloured monocultures (Fig.1a). It is caused by absorption of light by *BChl d* or *e* molecules located in bacterial chlorosomes (so called chlorosomal *BChl*).

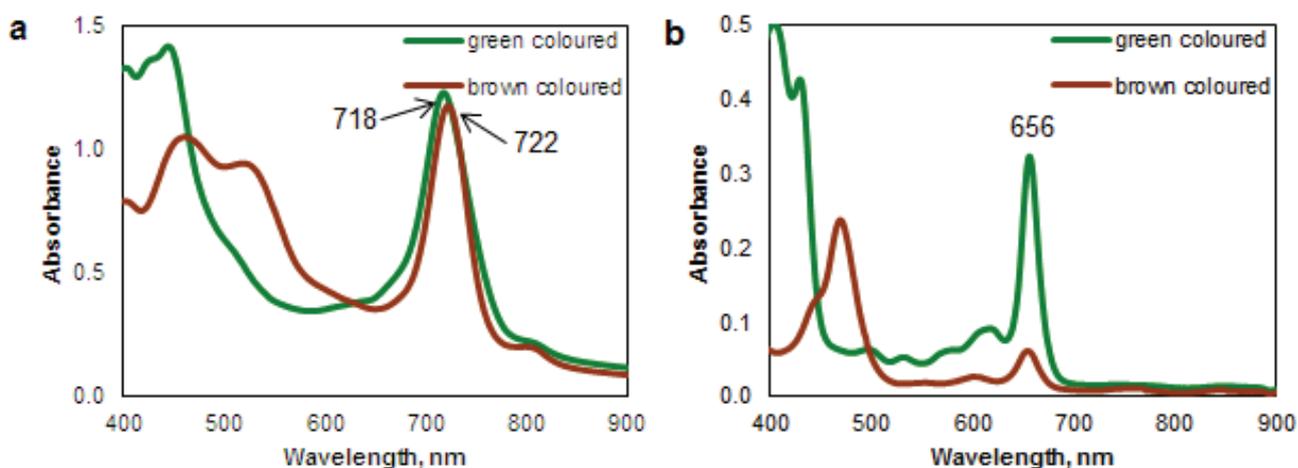


Figure 1: Absorbance spectra of green sulphur bacteria monocultures in water (a) and in acetone-methanol extract (b). The concentration of *BChl* was 15900 mg/m^3 for green-coloured GSB and 2700 mg/m^3 for brown-coloured GSB.

The absorption bands in the blue region are attributed as the Soret bands of *BChl* (from bacteria with both types of pigmentation) overlapped in case of green-coloured cultures with absorption by carotenoid chlorobactene. In brown-coloured cultures a distinct absorption band appears around 525 nm due to the carotenoid isorenieratene. The absorbance values depend on the total content of *BChl* in the sample.

Pigment extraction and quantification

The extracts of *BChl* in the acetone-methanol mixture are true pigment solutions due to the fact that the solvent dissolves the cytoplasmic membrane of bacteria, penetrates inside the cell and dissolves the lipid component of the chlorosomal membrane.. Absorbance spectra of *BChl* consist of two peaks. The short wavelength peak, the Soret band, is located at 435 nm for *BChl d* and at 475 nm for *BChl e*. The long wavelength peak of both *BChl d* and *e* has a maximum at 654-656 nm (Figure 1b). It is that same peak used to calculate the total *BChl (d+e)* concentration according to the photometric equation (14, 15, 16):

$$[BChl (d + e)] = \frac{1.135 \cdot (D655 - D850) - 0.643 \cdot (D667 - D850) + 0.005}{V \cdot d \cdot \varepsilon(BChl d)} \cdot 10^6 v$$

The constant 0.005 related to the instrument baseline calibration was presented in the original formula of Overmann and Tilzer. We transformed the formula to fit the zero-baseline offset:

$$[BChl (d + e)] = \frac{1.135 \cdot (D655 - D850) - 0.643 \cdot (D667 - D850)}{V \cdot d \cdot \varepsilon(BChl d)} \cdot 10^6 v$$

where:

- $[BChl (d + e)]$ is in mg/m³
- $D655$, $D667$ and $D850$ are the absorbances at 655, 667 and 850 nm
- v is the volume of acetone-methanol extract in ml
- V is the volume of the water sample in ml
- d is the optical path length in cm
- $\varepsilon(BChl d) = 98.0 \text{ cm}^2/\text{mg}$ is the extinction coefficient for *BChl d*.

Subtraction of absorbance at 850 nm was used to take into account wavelength-independent scattering due to bacterial particles in extracts.

Measurements in water

Since our aim was to evaluate *BChl* pigments for the cells directly in water, we calculated the area ΣD under the long-wave peak of the GSB absorption spectrum to estimate the total *BChl* concentration (Figure 2). To do this, we summed up all the absorbances in the spectral range of 650-800 nm with 1 nm interval and subtracted the trapezoid baseline resulting from light scattering on bacterial cells.

When we plotted the absorbance peak area ΣD versus *BChl* concentration, we found that the area of the far-red peak in the absorption spectrum of the aqueous sample is proportional to the *BChl* concentration calculated from the extract as:

$$\Sigma D = k \cdot [BChl (d + e)],$$

where:

- ΣD is the absorbance peak area in nm, and
- $[BChl (d + e)]$ is the total bacteriochlorophyll *d* and *e* concentration in mg/m³.

The correlation was good for both cultures but differed by pigmentation. The correlation coefficient was $R=0.97$ for green-coloured green sulphur bacteria, and $R=0.90$ for brown-coloured green sulphur bacteria (Figure 3). The regression slope was higher for brown-coloured monocultures

($k_{BChl e} \approx 0.0068 \text{ nm} \cdot \text{m}^3/\text{mg}$ with a correlation coefficient of $R=0.90$) than for green-coloured ones ($k_{BChl d} \approx 0.0032 \text{ nm} \cdot \text{m}^3/\text{mg}$). We explain this by the approximately two times higher molar extinction coefficient for *BChl d* than for *BChl e* (17), and hence underestimation of *BChl e* concentration using the simplified equation of Overmann and Tilzer for the total BChl (*d+e*) concentration.

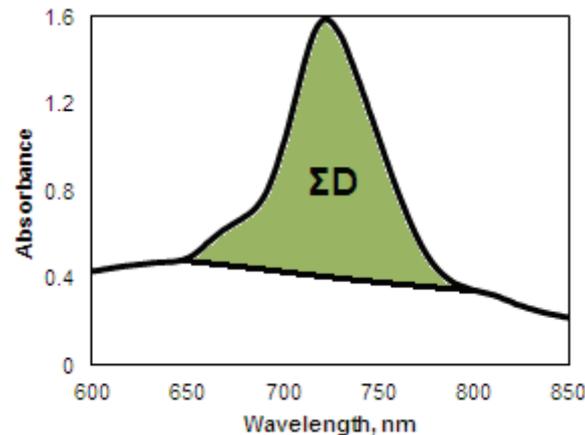


Figure 2: Calculation of the area under the far-red absorbance peak of *BChl* for intact cells of green sulphur bacteria.

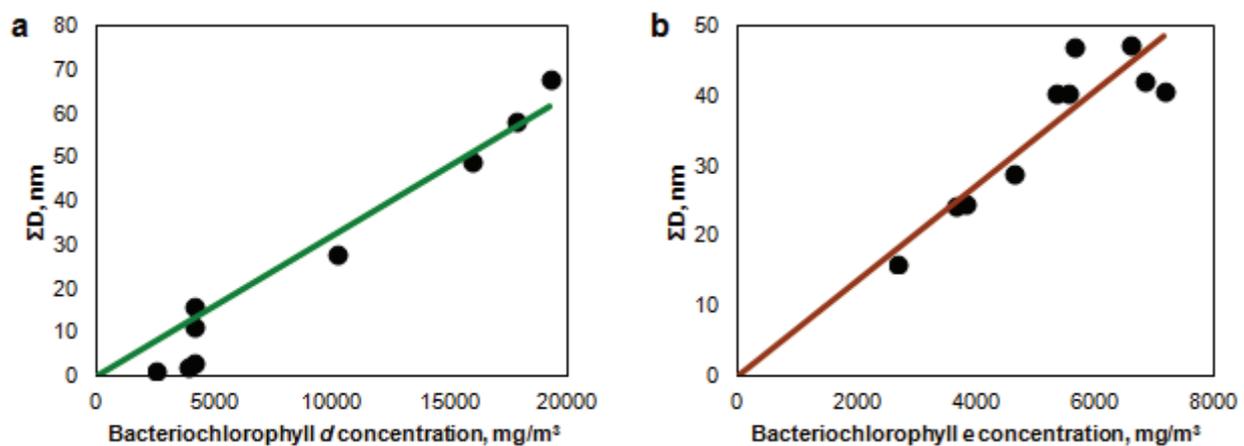


Figure 3: Dependences of the area ΣD under the far-red absorbance peak of *BChl* in intact bacterial cells versus *BChl* concentration estimated by pigment extraction for green-coloured monocultures (a) and brown-coloured monocultures (b) of green sulphur bacteria.

We analysed absorbance spectra of water samples collected in the lake Trekhtzvetnoe near the chemocline. Figure 4 shows the depth distribution of the area ΣD of the far-red *BChl* absorption in intact bacterial cells and its dependence on the pigment concentration. Though the maximal *BChl* concentration was approximately twice as high in July as in March 2017, practically all points on the Figure 4b and 5b fit the same regression line estimated for the green-coloured GSB monoculture (see Figure 3a). This fact indicates that near the chemocline of lake Trekhtzvetnoe in studied seasons only green-coloured bacteria were living, which agrees with our previous studies of the same lake using fluorescence techniques (18, 19, 20).

DISCUSSION

It is suggested to evaluate the *BChl* concentration in water samples from absorbance measurements directly in water and to calculate the pigment concentration according to the formulae:

$$[BChl d] = 312.5 \Sigma D \text{ for green-coloured GSB,}$$

$$[BChl e] = 147.1 \Sigma D \text{ for brown-coloured GSB,}$$

where the concentrations are in mg/m^3 , ΣD is the far-red *BChl* absorbance peak area determined within the spectral range of 650-800 nm after subtraction of the scattering baseline. The coefficients 312.5 and 147.1 are reverse values of the regression slopes $k_{BChl_e} \approx 0.0068 \text{ nm}\cdot\text{m}^3/\text{mg}$ and $k_{BChl_d} \approx 0.0032 \text{ nm}\cdot\text{m}^3/\text{mg}$.

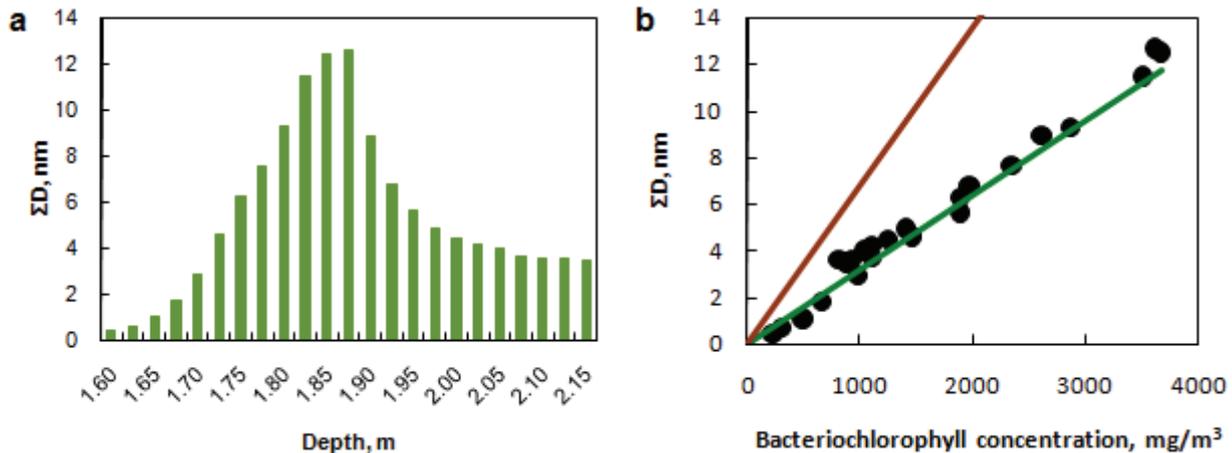


Figure 4: Depth distribution of the peak area ΣD for the far-red *BChl* absorption (a) and dependences of ΣD on *BChl* concentration (b) for water in the lake Trekhtzvetnoe in March 2017. Green and brown lines correspond to regression lines (Figure 3a, 3b) for green-coloured and brown-coloured monocultures respectively.

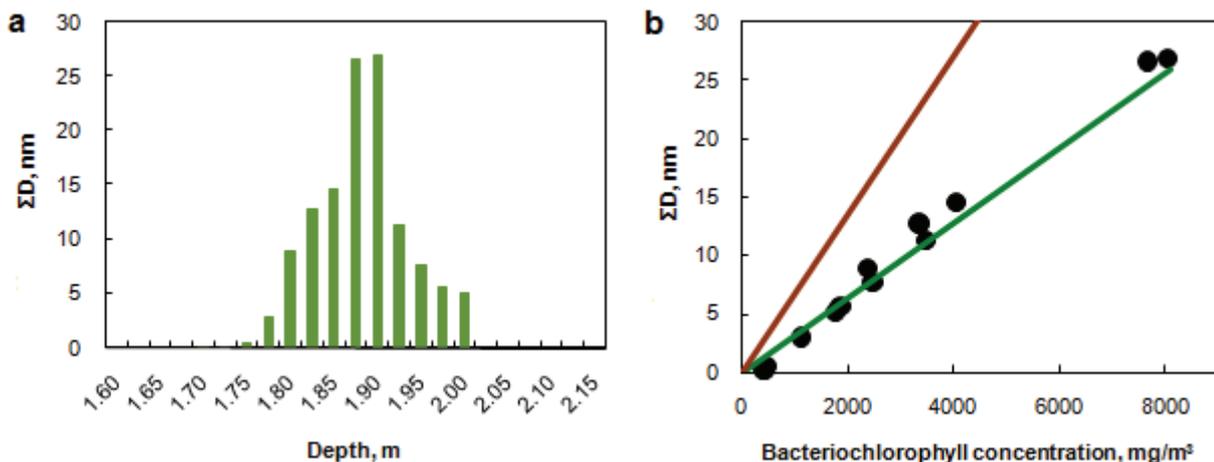


Figure 5: Similar graphs as in Figure 4 plotted for water in the lake Trekhtzvetnoe in July 2017.

The suggested method can be used in communities where only one type of microorganisms predominates (either green-coloured or brown-coloured GSB). This technique has apparent advantages compared to the traditional spectrophotometric evaluation of pigments using extractions in organic solvents. Absorption measurements performed directly on water samples containing living bacteria are easier and faster to accomplish; there is no need for the usage of chemicals. Moreover, *BChl* and *Chl* absorption bands are located in different spectral regions if measured on intact cells in water (positioned around 725 and 660 nm correspondingly), but hardly distinguished in extracts in organic solvents (overlapping peaks with maxima at 655 for chlorosomal *BChl* and 663 nm for *Chl* a). We believe that in the future the described spectrophotometric method of *BChl* quantification without any sample pre-treatment will be performed *in situ* with submersible spectral instruments.

However, this method cannot be used in highly turbid waters due to dominating scattering in the measured spectrum. The suggested algorithm can be utilised for water samples if *BChl* absorption is higher than the scattering contribution within the spectral range 650-800 nm. A small integrating

sphere can also be used to avoid the effect of scattering. In other words, this method can be used if the BChl far-red peak area ΣD shown in Figure 2 as green area exceeds the trapezoid scattering lying below the peak.

The minimal and maximal *BChl* concentrations determined with a spectrophotometer for undiluted samples placed in a cuvette with 1 cm optical path length can be estimated as follows: the smallest *BChl* concentration can be measured at absorbances $D \approx 0.01$, the highest concentration at $D \approx 2$. This corresponds to *BChl* concentrations from about 400 mg/m³ to 100 g/m³. As a rule, higher *BChl* concentrations can not be achieved in monocultures or in natural water due to lack of nutrients and illumination within so dense cultures. Otherwise, in laboratory measurements one can dilute initial samples or use a shorter optical path. For small concentrations of bacteria in water one can use cuvettes with a longer optical path length, from 2 to 5 cm.

CONCLUSIONS

We performed the research on absorption spectra of two groups of green sulphur bacteria (*Chlorobiaceae*) differing in pigmentation, green-coloured and brown-coloured. The far-red peak with a maximum at 720-725 nm attributed to chlorosomal *BChl* (both *BChl d* and *e*) in the intact cells of green sulphur bacteria had practically the same shape and position for all the studied cultures and in natural water samples. Its area (ΣD) in absorbance spectra of aqueous samples was calculated and compared with the total *BChl* (*d+e*) content estimated using pigments' extraction by organic solvent performed on the same specimen. Dependences of the area ΣD versus pigment concentration calculated from extraction using the formula of Overmann and Tilzer were plotted separately for green-coloured and brown-coloured bacteria in water. For each group of bacteria, it was found a good linear correlation of ΣD and *BChl* (*d+e*) concentration. The regression slope was higher for brown-coloured ($k_{BChl d} \approx 0.0032 \text{ m}^3/\text{mg}$) than for green-coloured species ($k_{BChl e} \approx 0.0068 \text{ m}^3/\text{mg}$) in monocultures of green sulphur bacteria. We explain this by the higher molar extinction coefficient for *BChl d* than for *BChl e*. The photometric equations determined using the regression slopes allow for quantification of *BChl* in living bacterial cells directly in water without any sample pre-treatment, and potentially to use the spectrophotometric method *in situ* with submergible spectral instruments used in communities where only one type of microorganisms predominates (either green-coloured or brown coloured GSB).

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