

Original paper

Methodological Features in Measuring the True Light Absorption Spectrum of Monocultures

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Abstract

This study presents a technique for determining the true light absorption spectrum of a dense culture of the marine coccolithophore *Chrysothila* sp. using a single-beam MS 122A spectrophotometer equipped with an integrating sphere. The main problem with standard measurements is the distortion of the spectrum due to light scattering by cells, which is especially noticeable in the near-infrared region (750–800 nm), where the pigments are not absorbed, but the signal is not zero. To compensate for the scattering effect, the authors used an approach based on recording absorption spectra at two positions from the integrating sphere: standard (close to) and at a distance of 2 mm. The correction factor, independent of wavelength, was calculated from data in the range of 750–800 nm. Its value was 3.77. The true absorption spectrum, stripped of the scattering contribution, was calculated using the proposed formula. The technique has shown its effectiveness for cultures with a high cell density, providing zero absorption values in the near-infrared region. However, with a low cell concentration, the technique is inapplicable due to a significant increase in errors. Thus, the work demonstrates a practical way to correctly determine *in vivo* absorption spectra using available equipment, which is important for ecological and physiological studies of phytoplankton as well as for development of regional remote sensing algorithms.

Keywords: spectrophotometry, integrating sphere, light absorption, coccolithophorides, *Chrysothila* sp., true absorption spectrum, correction factor, *in vivo*, *in vitro*, acetone extract

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Методические особенности измерения истинного спектра поглощения света монокультурами

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Аннотация

Представлена методика определения истинного спектра поглощения света плотной культурой морской кокколитофориды *Chrysolita* sp. с использованием однолучевого спектрофотометра МС 122А, оснащенного интегрирующей сферой. Основная проблема стандартных измерений заключается в искажении спектра из-за светорассеяния клетками, что особенно заметно в ближней инфракрасной области (750–800 нм), где поглощение пигментов отсутствует, но измеряемый сигнал не равен нулю. Для компенсации эффекта рассеяния использован подход, основанный на регистрации спектров поглощения при двух положениях кюветы относительно интегрирующей сферы: стандартном (вплотную) и на расстоянии 2 мм. Поправочный коэффициент, не зависящий от длины волны, рассчитывали по данным в области 750–800 нм, его значение составило 3.77. Истинный спектр поглощения, очищенный от вклада рассеяния, вычисляли по предложенной формуле. Методика показала эффективность для культур с высокой численностью клеток, обеспечивая нулевые значения поглощения в ближней ИК-области. Однако при низкой концентрации клеток метод неприменим из-за значительного роста погрешностей. Таким образом, работа демонстрирует практический способ корректного определения спектров поглощения *in vivo* на доступном оборудовании, что важно для экологических и физиологических исследований фитопланктона, а также для развития региональных алгоритмов дистанционного зондирования.

Ключевые слова: спектрофотометрия, интегрирующая сфера, поглощение света, кокколитофориды, *Chrysolita* sp., истинный спектр поглощения, поправочный коэффициент, *in vivo*, *in vitro*, ацетоновый экстракт

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Introduction

Spectrophotometers are the primary tool for assessing the optical properties of photosynthetic cells. However, the reliability of *in vivo* absorption spectrum measurements is limited by the distorting effect of light scattering, the magnitude of which depends both on the distribution of scattered light within the sample and on the geometry of the cuvette and detector.

The most effective way to minimize the loss of scattered light is to use an integrating sphere (IS) in spectrophotometers [1], the inner surface of which has a high reflection coefficient. When the studied sample is placed inside such a sphere, all scattered light reaches the photodetector, and the measured optical density is determined only by the true absorption of light by the sample [2]. However, in standard spectrophotometers equipped with an IS, the design typically does not allow the sample to be placed inside the sphere, instead, the cuvette is positioned in front of the entrance port. In this configuration, it is impossible to completely eliminate the contribution of scattering.

Two approaches for correction have been proposed in the literature: the use of correction factors and the method of recording spectra at different distances from the IS [3, 4]. The second approach is based on the fact that changing the distance to the IS allows the fraction of scattered light in the recorded signal to vary. Measurements at two distances (one close to and one far from the IS) make it possible to reconstruct the absorption spectrum compensated for scattering, which is confirmed by values approaching zero in the 750–800 nm range, where pigment absorption is absent [5].

Subsequently, such corrected spectra can be used in regional algorithms to separate the contributions of coccolithophores, diatoms, and dinoflagellates from multispectral measurements of the sea surface reflectance coefficient [6], obtained, for example, from satellite data.

For the Black Sea, an urgent task is the development of regional algorithms for interpreting satellite data, which allow the separation of the contribution of various taxonomic groups of phytoplankton (coccolithophores, diatoms, and dinoflagellates) to the total biomass [7]. Solving this problem requires reference absorption spectra of pure cultures of dominant species. In 2023, a series of studies was initiated to investigate the spectral features of the light absorption coefficient of individual Black Sea phytoplankton species of different taxonomic affiliations. However, the quality of the initial *in vivo* spectra obtained using the MS 122A spectrophotometer by the standard method remains unsatisfactory for reliable separation of these contributions, making scattering correction mandatory.

The present paper aims to determine the true light absorption spectrum of a batch culture of the marine coccolithophore *Chrysotila* sp. using a single-beam MS 122A spectrophotometer and the method of measurements at two distances from the IS. This work was carried out using materials from a report presented at the XIII All-Russian Conference with International Participation “Current Problems in Optics of Natural Waters 2025” [8].

Materials and methods

The study used an algologically pure culture of the coccolithophore *Chrysotila* sp. P. L. Anand from the collection of living cultures of marine planktonic microalgae at A. O. Kovalevsky Institute of Biology of the Southern Seas of RAS.

The algae were cultivated in 0.2 L conical flasks in $f/2$ medium [9] prepared with pasteurized seawater. To maintain the culture in the exponential growth phase and at a constant cell density, it was diluted daily with fresh nutrient medium. The cultivation temperature was $18 \pm 1^\circ\text{C}$, which corresponds to the temperature optimum for the studied species.

Absorption spectra were recorded in cuvettes with an optical path length of 1 cm in the range from 300 to 800 nm with a step of 1 nm using a single-beam MS 122A spectrophotometer (SOL instruments, Minsk, Belarus), equipped with a diffuse transmission and reflection attachment with an IS. During the measurements, the cuvette was placed in the cuvette holder of the attachment in front of the IS entrance port¹⁾. The inner diameter of the IS is 50.8 mm (2 inches). The inner surface coating of the IS is BaSO₄.

Measurements were carried out at two cuvette positions (Fig. 1): the standard position – flush against the IS entrance port ($r = 0$); the distant position – at the maximum possible distance for this setup, $r = 2$ mm from the IS entrance port. The design of the cuvette compartment of the MS 122A spectrophotometer does not allow the cuvette to be placed at a distance greater than 2 mm (a limitation imposed by the mirror in the optical system); therefore, this value was used in the work.

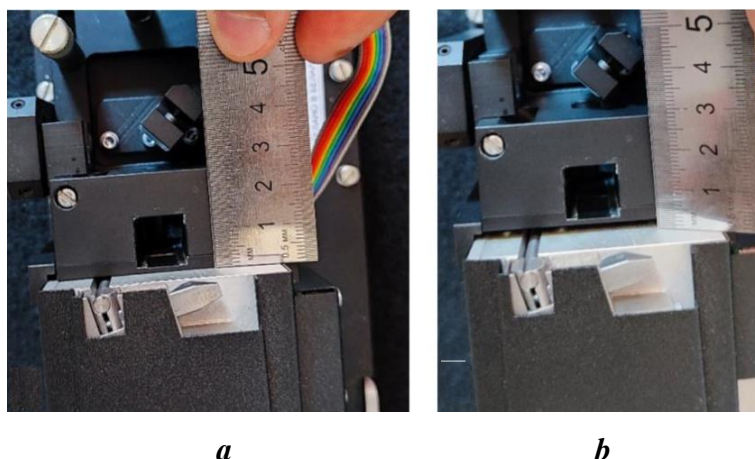


Fig. 1. Top view of the MS 122A cuvette compartment: *a* – in a position close to the integrating sphere (IS); *b* – at a distance of 2 mm from the IS

¹⁾ Available at: <https://solinstruments.by/produkcija/spektrofotometri/mc-122/dopolnitelnoe-oborudovanie/> [Accessed: 14 February 2025].

The true absorption spectrum, compensated for scattering, was determined by the formula [1]:

$$\tilde{a}(\lambda) = a_{\text{ph}}(\lambda; r) - L_{\text{att}}(r; 0) \cdot [a_{\text{ph}}(\lambda; r) - a_{\text{ph}}(\lambda; 0)]. \quad (1)$$

where $a_{\text{ph}}(\lambda; r)$ is the light absorption coefficient of the sample located at a distance r from the IS; $L_{\text{att}}(r; 0)$ is the correction factor; and $a_{\text{ph}}(\lambda; 0)$ is the light absorption coefficient at the standard cuvette position.

Experiments studying the influence of the distance from the IS to the sample on absorption spectra are well described in [3].

It is assumed that the correction factor $L_{\text{att}}(r; 0)$ is wavelength-independent and its value can be determined by considering the wavelength region where the sample does not absorb light (i.e., the 750–800 nm region of the visible range):

$$L_{\text{att}}(r; 0) = \frac{a_{750-800}(\lambda; r)}{a_{750-800}(\lambda; r) - a_{750-800}(\lambda; 0)}. \quad (2)$$

For comparative analysis, pigments were extracted from the cells with 100% acetone according to the method described in [10]. Absorption spectra of the extracts were recorded on the same instrument using standard 1 cm cuvettes.

Results and discussion

With the standard cuvette position (close to the IS), light absorption by the sample $a_{\text{ph}}(\lambda; 0)$ in the 750–800 nm range, where pigments are not absorbing, does not reach zero values. In this region, light absorption increases monotonically with increasing distance between the cuvette and the IS. This phenomenon is caused by non-selective scattering by microbial cells, which arises from abrupt changes in the refractive index at interfaces. Selective scattering likely also contributes, associated with abrupt changes in the refractive index caused by pigments embedded in thylakoid membranes within the spectral regions of their light absorption [4].

Fig. 2 shows the light absorption spectra of a dense culture obtained with the MS 122A spectrophotometer at two cuvette positions: the standard position, $a_{\text{ph}}(\lambda; 0)$, and the position at a distance of 2 mm from the IS entrance port, $a_{\text{ph}}(\lambda; r)$.

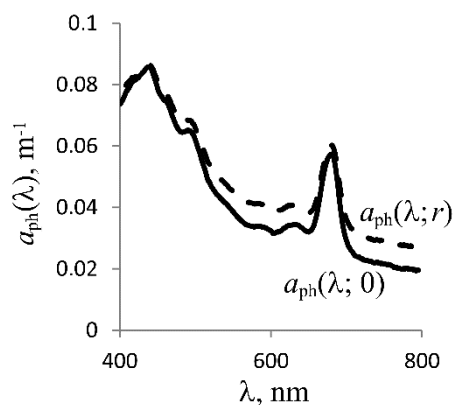


Fig. 2. Examples of light absorption spectra of the *Chrysolita* sp. culture obtained at the standard cuvette position, $a_{\text{ph}}(\lambda; 0)$, and at a distance of $r = 2$ mm from the IS input window, $a_{\text{ph}}(\lambda; r)$.

To correct for the scattering contribution, formula (2) was used, and calculations were performed for several λ values:

$$L_{750} = \frac{a(750; r)}{a(750; r) - a(750; 0)} = \frac{0.0289}{0.0289 - 0.0216} = 3.96,$$

$$L_{775} = \frac{a(775; r)}{a(775; r) - a(775; 0)} = \frac{0.0276}{0.0276 - 0.02} = 3.63,$$

$$L_{800} = \frac{a(800; r)}{a(800; r) - a(800; 0)} = \frac{0.0268}{0.0268 - 0.0196} = 3.72.$$

According to the methods described in [3], the correction factor L was determined as the average value in the 750–800 nm region. For the studied *Chrysolita* sp. culture, the average value of the coefficient $L_{\text{att}}(r; 0)$ for the three wavelengths was 3.77 in this work.

Using formula (1), the true absorption value was calculated for each wavelength. As an example, the calculation for the wavelength $\lambda = 443$ nm is presented:

$$\tilde{a}(443) = 0.0853 - 3.77 \cdot [0.0853 - 0.0841] = 0.081.$$

Carrying out similar calculations for the remaining wavelengths, the true spectrum of the light absorption coefficient of the *Chrysolita* sp. culture, compensated for scattering, was obtained (Fig. 3). A characteristic feature of this spectrum is the absence of light absorption in the 750–800 nm range (Fig. 3), which confirms the quality of the correction performed.

It should be noted that the described method is effective only at high cell densities, when the culture in the cuvette exhibits a noticeable color, distinguishable visually. At low algae concentrations, the recorded spectra contain (at $a_{\text{ph}}(750; 0) > 0.02 \text{ m}^{-1}$) a significant number of artifacts, which can be mistakenly interpreted as spectral peaks. An attempt to recover the true spectrum under such conditions leads to the amplification of noise spikes and an increase in measurement error. As an example, Fig. 4 shows spectra for two concentrations: the undiluted culture (570,000 cells/mL) (Fig. 4, *a*) and the culture after an eight-fold dilution (71,300 cells/mL) (Fig. 4, *b*).

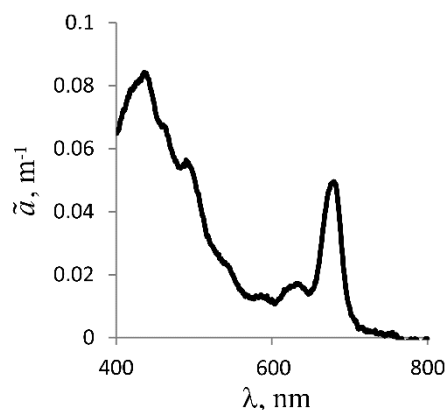


Fig. 3. True light absorption spectrum of the *Chrysolita* sp. culture determined by expression (1)

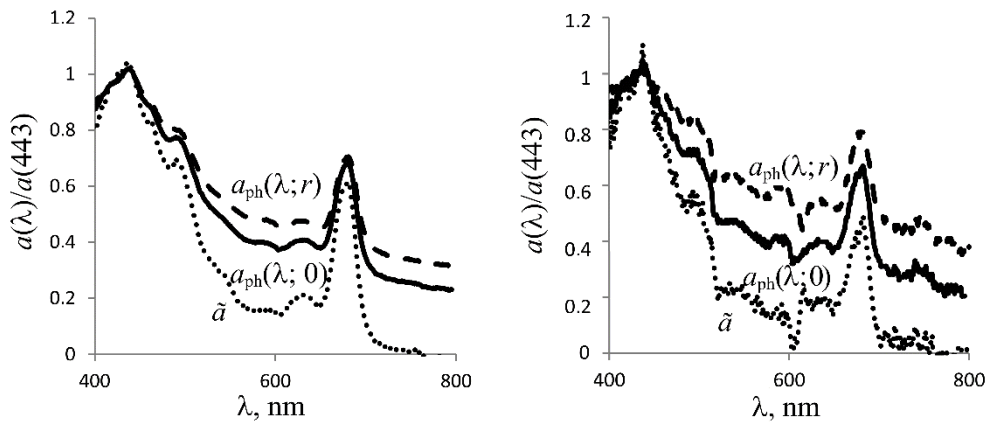


Fig. 4. Absorption spectra of the *Chrysofila* sp. culture normalized to 443 nm, obtained at the standard position of the cuvette, $a_{ph}(\lambda; 0)$, at a distance $a_{ph}(\lambda; r)$ from the IS input window and the true spectra \tilde{a} calculated using expression (1) for a concentration of 570,000 cells/mL (left) and 71,300 cells/mL (right)

Fig. 5 presents a comparison of the true *in vivo* absorption spectrum (dashed line) and the spectrum of the acetone extract (solid line) for the concentration of 570,000 cells/mL. The spectrum of the acetone extract of the algae is characterized by a shift of the peaks towards shorter wavelengths [11, p. 304] relative to the *in vivo* spectrum. This shift is mainly due to the destruction of pigment-protein complexes during extraction with organic solvents, changes in the polarity of the medium, and aggregation/dissociation of pigments in acetone.

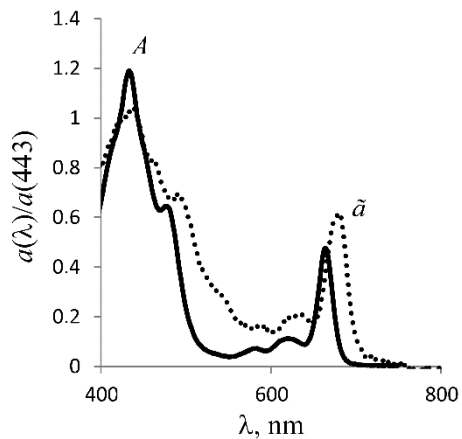


Fig. 5. Light absorption spectra of the *Chrysofila* sp. culture normalized to 443 nm, where the dotted line is the true spectrum calculated using expression (1), the solid line is the spectrum of the acetone extract

This fact is critically important for interpreting remote sensing data, since the calibration of satellite instruments is often based on *in vitro* spectra, whereas *in situ* measurements correspond to the *in vivo* state of pigments. The difference in spectral characteristics is due to the fact that in acetone extracts, some pigments (e. g., chlorophylls) can form aggregates (dimers, oligomers). Aggregation often leads to splitting and shifts of absorption peaks. In the native state, the absorption maximum of chlorophyll is around 660–680 nm (red region), while in acetone extracts the peak can shift to 650–665 nm (hypsochromic shift), which is associated with the loss of interaction with proteins [7].

Conclusions

A method for correcting phytoplankton absorption spectra for scattering has been adapted for the single-beam MS 122A spectrophotometer with an IS. It was established that the maximum possible distance of the cuvette from the IS (2 mm) is sufficient for calculating the correction factor and obtaining the true absorption spectrum, confirmed by values approaching zero in the 750–800 nm range.

For the *Chrysothila* sp. culture, the average value of the correction factor $L = 3.77$ was calculated in the 750–800 nm region. Using this factor, the true *in vivo* absorption spectrum, corrected for the scattering contribution, was obtained.

The applicability limits of the method were determined: it is effective only at high cell densities, when the absorption coefficient at a wavelength of 750 nm $a_{ph}(750; 0)$ exceeds 0.02 m^{-1} . At low concentrations (below 700,000 cells/mL), correction leads to noise amplification and the appearance of artifacts.

It was confirmed that the spectra of acetone extracts of *Chrysothila* sp. pigments are characterized by a hypsochromic shift (shift to shorter wavelengths) relative to *in vivo* spectra, which is caused by the destruction of pigment-protein complexes and pigment aggregation in acetone. This difference must be taken into account when calibrating algorithms for remote sensing of phytoplankton from satellite data.

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Lyudmila V. Stelmakh – formulation and statement of the problem, concept development, analysis of the results, manuscript writing, study supervision

Natalya V. Minina – collection of study materials, primary processing and sorting of data, discussion of the results

Olga S. Alatartseva – collection of study materials, primary processing and sorting of data, discussion of the results

All authors have read and approved the final version of the manuscript.