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Application of structural, functional, fluorescent, and cytometric indicators for assessing physiological state of marine diatoms under different light growth conditions

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Abstract: The changes in the main structural, functional, fluorescent, and cytometric indicators of diatom microalgae *Phaeodactylum tricornutum* (Bohlin, 1897), *Nitzschia* sp., and *Skeletonema costatum* (Cleve, 1873) under different light growth conditions were analyzed; the potential of their application as possible indicators for monitoring algae physiological state was evaluated. For all studied species, uniform light dependences of specific growth rate, C/Chl *a*, coefficient of cell size variability, relative variable chlorophyll *a* fluorescence (Fv/Fm), FDA fluorescence, and ratio of living cells were obtained. A significant correlation was established between Fv/Fm and C/Chl *a* in algae cells. As shown, Fv/Fm indicator is ineffective for diagnosing changes in algae growth characteristics, when changing light conditions. Under optimal light conditions, the ratio of living cells in a population is at least 75%, and cell size variability (CV) is below 30%. In turn, a decrease in the ratio of living cells and an increase in CV correlate with a decrease in algae specific growth rate and an increase in C/Chl *a* in their cells under photoinhibition. The practicability of using FDA fluorescence and the ratio of living cells in unicellular algae cultures to assess a lethal effect of external factors on algae structural and functional characteristics is shown, since a drop in values of the described indicators is observed at extreme values of the light factor, which are lethal or close to them.

Key words: Microalgae, light intensity, flow cytometry, specific growth rate, C/Chl *a*, relative variable chlorophyll *a* fluorescence, FDA fluorescence, ratio of living cells, cell size variability

1. Introduction

Phytoplankton is a basic component of aquatic ecosystems, which determines the productivity of all links of the trophic chain and plays a key role in the global cycles of carbon, nitrogen, and phosphorus. A rapid response of microalgae to changes in environmental conditions allows using changes in their structural and functional characteristics as sensitive indicators of the ecological state of aquatic biocenoses. In turn, the use of modern research methods —flow cytometry in combination with using various vital dyes (Cid et al., 1996; Davey and Kell, 1996; Agustí and Sánchez, 2002), as well as biophysical method for recording the indicators of the variable chlorophyll *a* fluorescence (Owens, 1991; Falkowski and Kolber, 1995; Antal et al., 2001; Pogosyan and Matorin, 2005)— makes it possible to quickly track the effects of fast-flowing environmental impact on the algae functioning and their production potential. However, it is impossible to apply fluorescent and cytometric indicators for express diagnostics of algae functional state without understanding the qualitative and quantitative relationships between them and the main algae structural and functional characteristics (growth rate, pigment concentration, and ratio of the main intracellular components).

It is known that the stressful effect of an external factor does not always result in microalgae death; cells are capable of adapting to changing growth conditions by changing the ratios between the main intracellular components, enzyme systems activity, enzyme processes rate, cell morphology, and cell internal structure (Davey and Kell, 1996; Popova et al., 2004; Shoman and Akimov, 2013). In this case, the rate of photosynthesis and population growth may remain unchanged. And vice versa, the lack of algae growth, resulting from exposure to factors of various nature, cannot be unambiguously regarded as the population death: when exposed to favorable conditions, algae functional characteristics can be restored, and cell growth can be resumed (Solomonova and Akimov, 2014). Under increasing anthropogenic load on marine biocenoses, the issue of assessing lethal effects, leading to irreversible consequences in the restructuring of ecological niches of marine ecosystems, remains particularly relevant.

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In view of the above, the aim of this study was to identify patterns of change in the main structural, functional, fluorescent, and cytometric indicators of diatoms (*Phaeodactylum tricornutum*, *Nitzschia* sp., and *Skeletonema costatum*) under various light growth conditions, as well as to evaluate the effectiveness of the application of fluorescent and cytometric indicators for quick diagnosis of algae functional state in culture.

2. Materials and methods

As the object of research, monospecies cultures of marine diatoms were used: *Phaeodactylum tricornutum* (Bohlin, 1897), *Nitzschia* sp., and *Skeletonema costatum* (Cleve, 1873) from the collection of Algae Ecological Physiology Department of A. O. Kovalevsky Institute of Biology of the Southern Seas of RAS. These species were chosen, since the first two of them are distinguished by a high viability during cultivation and are often used in hydrobiological studies as model objects. Species *Nitzschia* sp. isolated from the plankton of the Black Sea, it differs from the known species of the genus *Nitzschia* in its small size (length 17–20, width 2–2.5 microns) and fusiform shape with wide-rounded ends. And *S. costatum* is one of the most widespread representatives of the Black Sea phytoplankton in autumn, winter, and spring.

In the experiments, the algae were cultivated on the F/2 medium (Andersen, 2005). *P. tricornutum* and *Nitzschia* sp. were grown at irradiance of 4, 14, 25, 42, 85, 225, 430, 600, 900, and 1200 mkEm^{-2}s^{-1}. For *S. costatum*, a narrower irradiance range was chosen (5 to 530 mkEm^{-2}s^{-1}), which is due to low tolerance of the species to high light intensity. Algae adaptation to the specified light conditions was carried out at a temperature of (20 ± 1) °C for a time period, sufficient to achieve a constant growth rate at given irradiance (3–7 days). During the adaptation, the density of cultures was maintained in the growth exponential phase by dilution with fresh medium once a day up to carbon concentration of 1–1.2 mgL^{-1} in the samples. The cultivation flasks were illuminated with white light-emitting diodes; different irradiance levels were provided by varying the distance to the light source and using neutral-density filter. The irradiance level was determined inside the flasks by a probing 4P sensor of a QSL-2101 quantometer.

Culture medium pH was of 8.2–8.5.

During the experiments, cell concentration in the cultures was maintained at approximately the same level in the phase of exponential growth by periodic (once a day or less often for poorly growing cultures) dilution with fresh medium.

Organic carbon content in the samples was calculated from the optical density of the cell suspension, measured at a wavelength of 750 nm on a SPh-26 spectrophotometer (Gevorgiz and Schepachev, 2008). The values of the optical density and carbon content, measured by gas-adsorption chromatography on a CHN analyzer, were previously calibrated for each species (Grasshoff et al., 1983). Calibration graphs are given in the publication (Shoman and Akimov, 2013). To verify the reliability of the obtained results for the samples, carbon content was measured in parallel by optical density and by gas-adsorption chromatography on a CHN-1 analyzer.

Microalgae specific growth rate was calculated from carbon increase in the sample according to the equation given in (Finenko and Lanskaya, 1971):

$$\mu = \frac{\ln C_t - \ln C_0}{t} \quad (1)$$

where m is specific growth rate, days^{-1}; C_0 and C_t are initial carbon concentration in the sample and its amount after time t, respectively, mgL^{-1}; t is time between measurements, days.

Chlorophyll *a* concentration in the sample was determined by the standard spectrophotometric method (Sirenko et al., 1975). Thus, a certain amount of algae suspension was filtered through glass microfiber filters GF/F (Whatman) with a diameter of 15 mm at a vacuum (<0.2 atm). The suspension, deposited on the filter, was extracted with a 90% acetone solution at a temperature of +8…+10 °C for 18–24 h. The resulting extract was clarified by centrifugation at 3000 rpm for 10 min. The optical density of acetone extracts was determined on a Specord UV-VIS double-beam spectrophotometer (Carl Zeiss Jena) prior and after acidification with 5% hydrochloric acid solution. Chlorophyll *a* concentration was calculated by the generally accepted formula (Jeffrey and Humphrey, 1975).

For cytometric analysis, 3-mL samples were taken from the cultivation flasks. The samples were examined using a Cytomics FC500 Flow Cytometer (Beckman Coulter, USA), equipped with a 488-nm single-phase argon laser, and the CXP software. Algae were stained with the vital dye fluorescein diacetate (FDA) according to the protocol, published in (Solomonova and Mikhanov, 2011). Metabolic activity and pigment concentration in the cells were evaluated on two parametric cytograms by FDA fluorescence (FL1 channel in the green area of the spectrum, 525 nm) and autofluorescence (FL4 in the red region of the spectrum, 675 nm) on dimensionless logarithmic scales. Figures can be found in the publication (Solomonova and Mukhanov, 2011); that work was performed with the participation of the authors. The mean values of accumulated FDA fluorescence per cell were calculated; the indicator was denoted as FDA_{fl}.

The number of living and dead cells was calculated from chlorophyll *a* autofluorescence (FL4 in the red region of

the spectrum, 675 nm). On Figure 1, a histogram is given, which shows distribution of particles in *P. tricornutum* culture; each point corresponds to a recorded event on a scatter plot (FS) and red autofluorescence (FL4). In this case, region A corresponds to algae cells and possibly to large cell fragments with undamaged pigments. In region B, chlorophyll-free residues or algae cell fragments (dead cells) are recorded.

The variability of cell volumes was determined on a flow cytometer by a direct light scattering (FS channel), which is used to determine the relative cell diameter (Shapiro, 2003). To estimate the level of cell size variability based on the coefficient of variation, the Mamaev scale was used (Mamaev, 1975).

Changes in the variable chlorophyll *a* fluorescence [maximum quantum efficiency of photosystem II (PS II)] were determined. In the literature, this indicator is defined as efficiency of primary charge separation, efficiency of using light quanta, etc. Hereinafter, it is referred to as the coefficient of the relative variable chlorophyll *a* fluorescence – Fv/Fm (Owens, 1991; Falkovsky and Kolber, 1995; Antal et al., 2001).

Prior measuring chlorophyll *a* fluorescence, the samples were kept in the dark for 15 minutes. The measurement was carried out on a MEGA-25m fluorometer with pulse modulation of exciting light at a wavelength of approximately 455 nm (Pogosyan and Matorin, 2005), developed at Biophysics Department of Biology Faculty of M. V. Lomonosov Moscow State University according the formula:

$$Fv/Fm = \frac{Fm - F0}{Fm} \quad (2)$$

where Fm is the maximum fluorescence after a series of light flashes, saturating the photosynthesis reaction centers; F0 is the fluorescence value at open reaction centers.

Statistical data processing was carried out using standard software packages Microsoft Excel 7.0, Statistica 5, Grapher 9, and SigmaPlot for a personal computer. All the experiments were performed in three biological and three analytical replicates. The significance of the differences between the mean values was estimated by the Student's t-test at $p < 0.05$.

3. Results

Based on the results of the experimental study, uniform light dependences of growth rate were obtained for three diatom species (Figures 2A–2C). Considering the nature of the light factor effect, every curve was conventionally divided into three areas: light range, limiting algae growth; optimal irradiance range for growth (light plateau); and photoinhibition area.

Under light-limiting conditions, algae specific growth rate increased linearly with irradiance increasing; under optimal light growth conditions, it reached maximum values: 1.4 days^{-1} for *P. tricornutum*, 1.9 days^{-1} for *Nitzschia* sp., and 1.85 days^{-1} for *S. costatum*. Growth light saturation

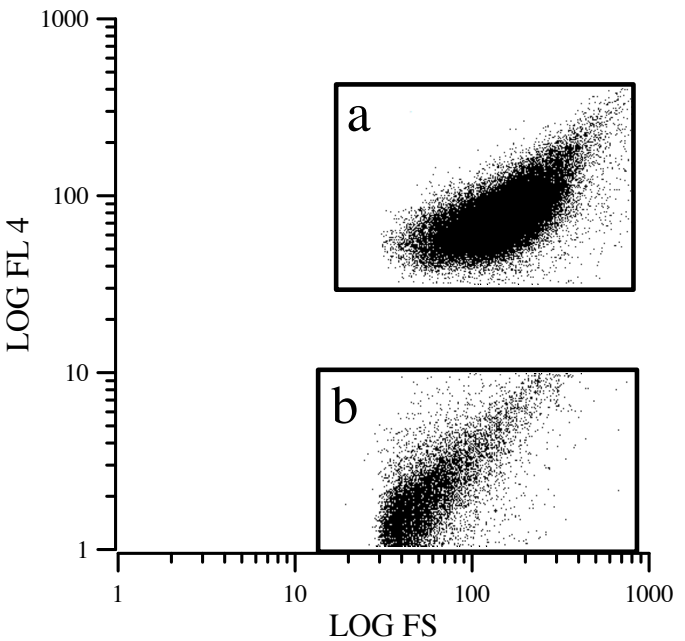

Figure 1. Distribution of suspended particles of *P. tricornutum* culture in the coordinates: direct light scattering (FS) and autofluorescence (FL4); a – a cluster, corresponding to the cells of the studied culture; b – particles with low pigment content or dead cells.

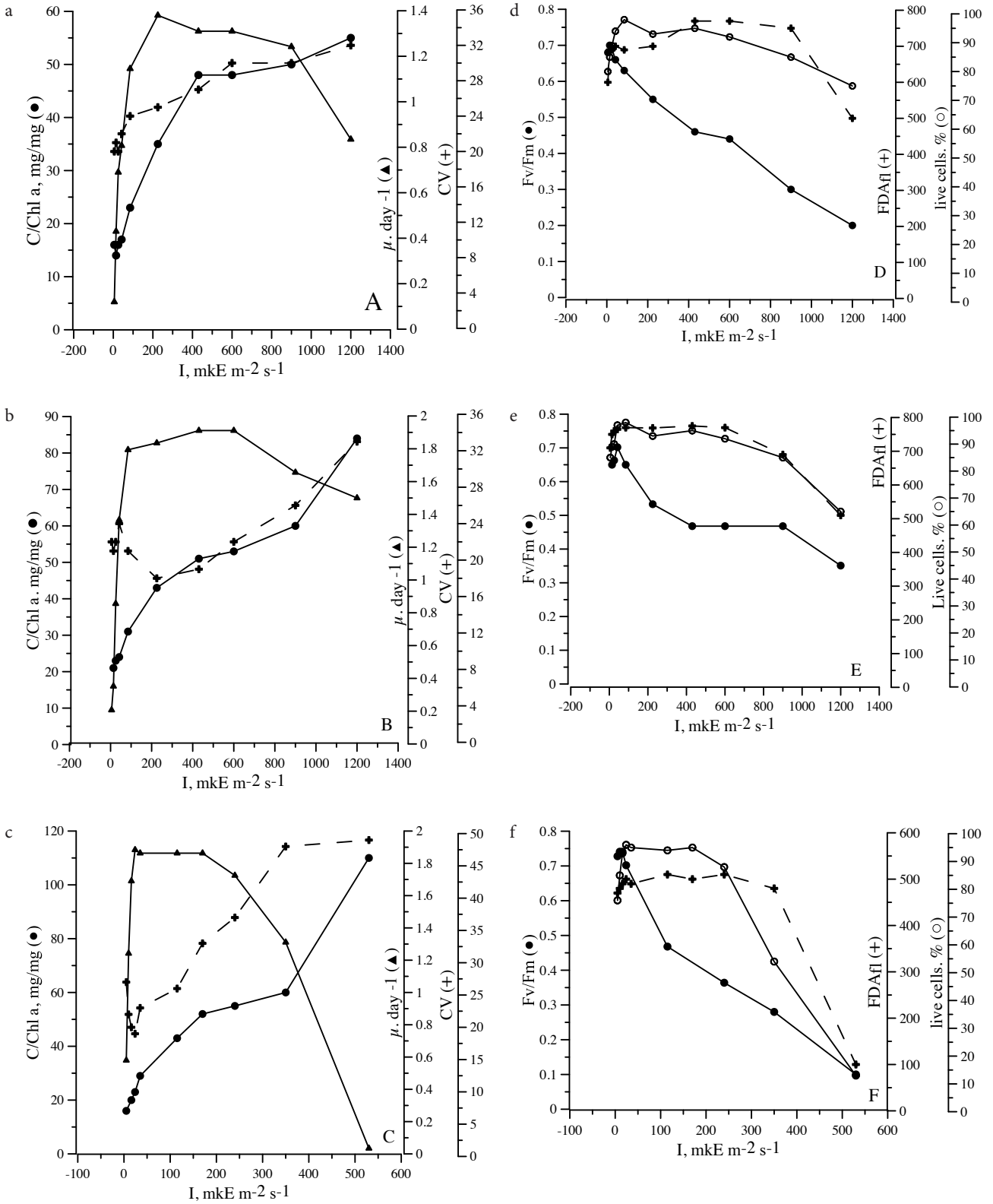

Figure 2. Change in specific growth rate, C/Chl *a*, coefficient of cell size variability, variable chlorophyll *a* fluorescence Fv/Fm, FDA fluorescence, and ratio of living cells in population, depending on light intensity, for *P. tricornutum* (A, D), *Nitzschia* sp. (B, E), and *S. costatum* (C, F).

(Ik) for *P. tricornutum* and *Nitzschia* sp. was recorded at light intensity of approximately 80 $mkEm^{-2}s^{-1}$. For *S. costatum*, μ-I dependence reached a plateau at much lower irradiance – 35 $mkEm^{-2}s^{-1}$. In total, the results of the experiment showed a high sensitivity of this species to the effect of high light intensity: photoinhibition of *S. costatum* growth was manifested even at irradiance above 280 $mkEm^{-2}s^{-1}$; at 530 $mkEm^{-2}s^{-1}$, algae died. Photoinhibition of *P. tricornutum* and *Nitzschia* sp. growth was observed at light intensities above 850 and 600 $mkEm^{-2}s^{-1}$, respectively.

In the range of irradiance, where light had no inhibitory effect on algae growth rate, the variability of intracellular carbon to chlorophyll *a* ratio (hereinafter C/Chl *a*) on irradiance was described by a hyperbolic dependence. At the same time, the boundaries of the ratio variability differed slightly between species. For *P. tricornutum*, C/Chl *a* increased 15 to 60 at a light range 14–900 $mkEm^{-2}s^{-1}$; for *Nitzschia* sp., the ratio increased 15 to 55 with an increase in irradiance 14 to 600 $mkEm^{-2}s^{-1}$; for *S. costatum*, C/Chl *a* increased 16 to 60 at 5–300 $mkEm^{-2}s^{-1}$. Under photoinhibition, all studied species showed a sharp increase in the intracellular carbon to chlorophyll *a* ratio, which correlates inversely with algae growth rate: in all cases, a slowdown in cultures growth was accompanied by an increase in C/Chl *a* values in their cells.

High values of the quantum efficiency of PS II reaction centers (Fv/Fm = 0.63…0.7) were maintained at 14–150 $mkEm^{-2}s^{-1}$ for *P. tricornutum* and *Nitzschia* sp. (Figures 2D and 2E). The recorded values of this indicator are close to the maximum ones, given in the literature, both for algae cultures and natural communities of phytoplankton, vegetating under optimal conditions (Kromkamp et al., 1998; Parhill et al., 2001). An increase in irradiance 150 to 600 $mkEm^{-2}s^{-1}$ led to a decrease in the relative variable chlorophyll *a* fluorescence values down to 0.45–0.55; at 800 $mkEm^{-2}s^{-1}$, Fv/Fm value was of 0.2–0.3. It should be noted that photoinhibition of *P. tricornutum* and *Nitzschia* sp. growth rates is recorded at irradiance above 600–900 $mkE{\cdot}m^{-2}{\cdot}s^{-1}$. For *S. costatum*, the maximum Fv/Fm values (0.7–0.73) were observed at a light intensity range 5–24 $mkEm^{-2}s^{-1}$ (Figure 2F). Cultivation of this species at 35–170 $mkEm^{-2}s^{-1}$ led to a gradual decrease in the relative variable chlorophyll *a* fluorescence, while a decrease in algae specific growth rate was registered at much higher irradiance values – above 280 $mkEm^{-2}s^{-1}$. At 530 $mkEm^{-2}s^{-1}$, there was a sharp and irreversible drop in the quantum efficiency of photosystem II (Fv/Fm) down to 0.1, which is typical for cells with deeply damaged pigment-protein complexes of PS II.

For *P. tricornutum* and *Nitzschia* sp., FDA_{fl} was weakly dependent on light intensity in the range 14–900 $mkEm^{-2}s^{-1}$. The decrease in FDA fluorescence at 1200 $mkEm^{-2}s^{-1}$ was observed; however, it was not as significant, as for the relative variable chlorophyll *a* fluorescence (Fv/Fm). For *S. costatum*, FDA fluorescence specific values remained at a high, relatively constant level at a light intensity range 5 to 350 $mkEm^{-2}s^{-1}$; further increase in irradiance up to 530 $mkEm^{-2}s^{-1}$ led to a sharp decrease in FDA_{fl} to minimum values, reflecting a lethal effect of given light intensity on algae functional state.

When cultivating algae under light-limiting conditions, the ratio of living cells in the suspension averaged 85% and did not differ significantly between species. The maximum ratio of living cells was observed under optimal light growth conditions. *P. tricornutum* and *Nitzschia* sp. cultivation at irradiance above 900 $mkEm^{-2}s^{-1}$ led to an increase in the number of dead cells in the cultures; their maximum ratio was recorded at 1200 $mkEm^{-2}s^{-1}$ – 40 and 35%, respectively. For *S. costatum*, a more significant accumulation of dead cells was observed at irradiance above 350 $mkEm^{-2}s^{-1}$. Light intensity of 530 $mkEm^{-2}s^{-1}$ led to *S. costatum* degradation, cell destruction, and death of the culture; the ratio of dead cells was 87%.

On Figures 3A and 3B, the cytograms are given of the distribution of the cell size of *P. tricornutum* culture, adapted to 225 $mkEm^{-2}s^{-1}$ (optimal light intensity) and 900 $mkEm^{-2}s^{-1}$ (inhibitory light intensity). A wider range of event distribution both in size and in fluorescence at high light intensity is noteworthy, compared to that of 225 $mkEm^{-2}s^{-1}$. High variability of these values in zone A, which corresponds directly to algae cells that retained photosynthetic pigments, indicates large physiological non-uniformity of the population; moreover, it may indicate deterioration in algae state. In this regard, we introduce an indirect indicator of algae functional state – the coefficient of variability of FS; this index of flow cytometer is applied to determine the relative cell diameter (Shapiro, 2003) (CV on the graphs). The results of the study showed as follows: under limiting and optimal irradiance, cell size variability, according to the Mamaev scale (Mamaev, 1975), was low for all studied species. CV increased significantly (up to 30%–40%) during culture adaptation to inhibitory irradiance, which is associated with change in algae physiological state and increasing ratio of dead cells. Dynamics of CV change for the studied algae species was identical.

Temporal dynamics of changes in specific growth rate is shown on Figure 4, as well as dynamics of Fv/Fm, FDA_{fl}, and C/Chl *a* of algae, when transferring from unfavorable light conditions (photoinhibition) to optimal ones. For *P. tricornutum* and *Nitzschia* sp. (Figure 4A), a decrease in all studied indicators was observed at inhibitory irradiance of 1200 $mkEm^{-2}s^{-1}$, but this decrease was reversible. When transferring these species to 200 $mkEm^{-2}s^{-1}$, the restoration of algae functional indicators was of a prolonged character. Thus, on the first day, a growth lag-phase was observed,

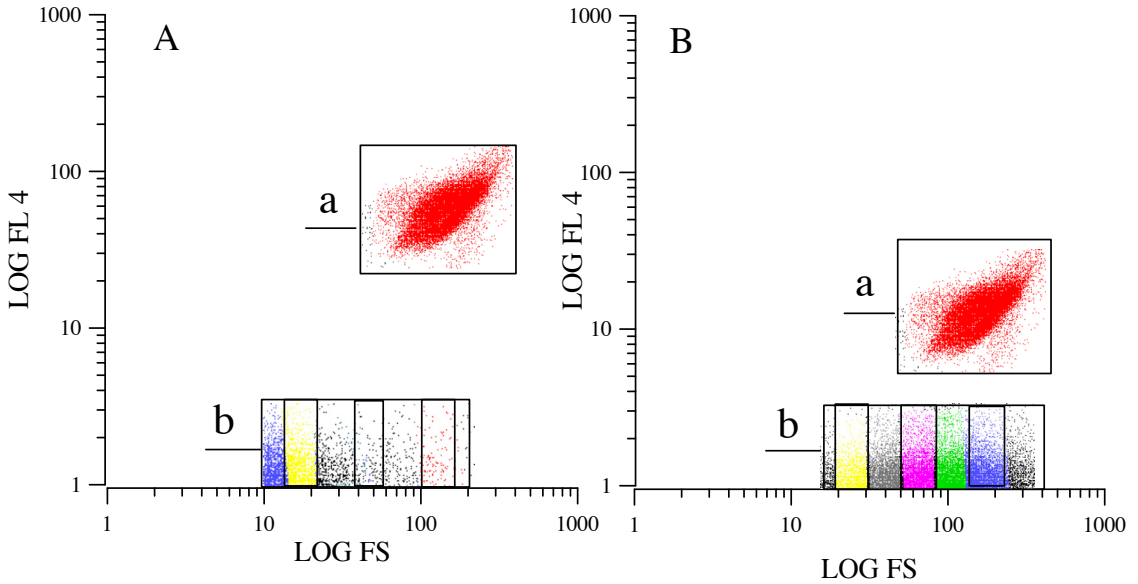

Figure 3. Cytograms of distribution of cell size of *P. tricornutum* culture, adapted to low (A) and high light intensity (B); a – a cluster, corresponding to the cells of the studied culture; b – particles with low pigment content or dead cells.

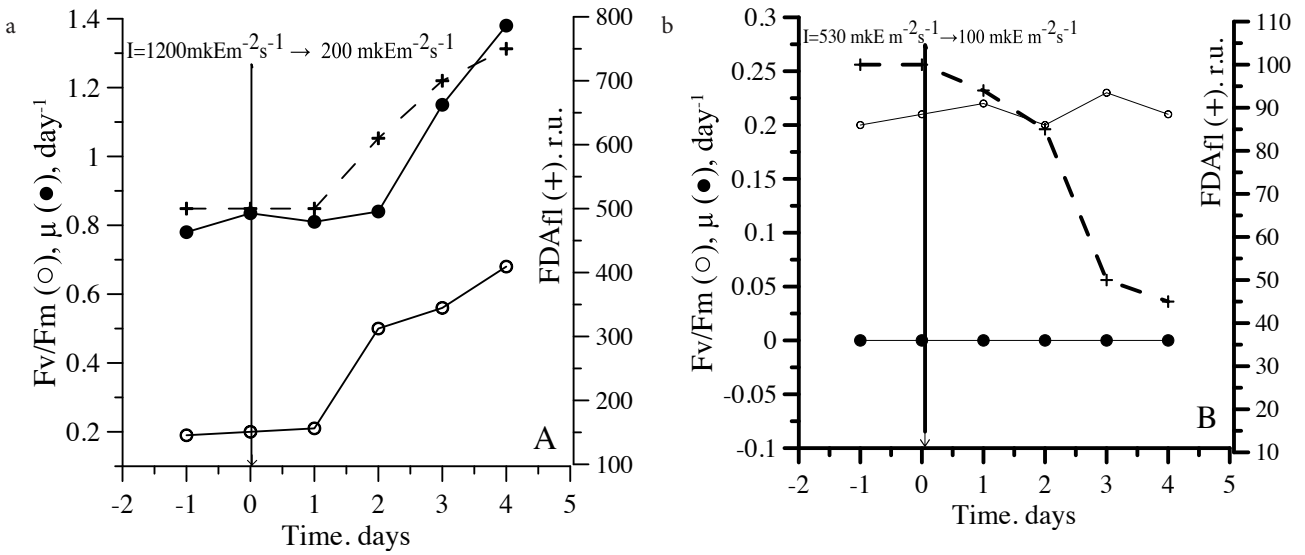

Figure 4. Dynamics of restoring *P. tricornutum* (A) and *S. costatum* (B) functional activity, when transferred from inhibitory irradiance to optimal one. The arrows indicate the beginning of the change in light conditions.

probably due to the low number of viable cells in the cultures. Further algae cultivation at 200 mkEm^{-2}s^{-1} led to restoration of their functional state and a change in specific growth rate, Fv/Fm, FDA$_{fl}$, and C/Chl *a*, corresponding to new light cultivation conditions. For *S. costatum*, the exposure to inhibitory irradiance of 530 mkEm^{-2}s^{-1} for 3 days led to death of the culture; cell elimination was observed with impossibility of subsequent recovery, when transferring algae to optimal light conditions – 100 mkEm^{-2}s^{-1} (Figure 4B). Sharp drop in FDA$_{fl}$ value at 530 mkEm^{-2}s^{-1} and impossibility of its recovery once again confirm the thesis, put forward previously (Solomonova and Akimov, 2014): using this marker of algae functional state allows to assess a lethal effect of environmental factors.

The correlation between intracellular C/Chl *a*, which reflects the specific chlorophyll *a* content per unit of algal biomass, and Fv/Fm was established according to

the results of the study (Figure 5A). An inverse linear correlation was obtained: Fv/Fm = − 0.007 × C/Chl *a* + 0.827. The maximum Fv/Fm values (0.7–0.75) are typical for cells with low intracellular carbon to chlorophyll *a* ratio (15–20). In turn, an increase in C/Chl *a*, caused by a light-dependent change in chlorophyll *a* content in algae, is accompanied by a linear decrease in Fv/Fm values down to zero, when the culture growth stops.

The study revealed no correlation between FDA fluorescence, algae growth rate, and C/Chl *a*. It was shown that a high level of esterase activity in cells is retained in a wide range of C/Chl *a* (Figure 5B). A drop in FDA_{fl} values down to 80 for *S. costatum*, corresponding values of C/Chl *a* (110), and $\mu \rightarrow 0$, as noted above, are observed at high light intensity of 530 $mkEm^{-2}s^{-1}$ and indicate a lethal effect of that irradiance on algae structural and functional characteristics.

The ratio of living cells in the culture was about 80%–100% for all algae species, when light did not inhibit algae growth, and the range of light-dependent changes in C/Chl *a* was of 15–60 (Figure 5C); in turn, the coefficient of variation of cell size CV was less than 30% (Figure 5D). A progressive decrease in the ratio of living (photosynthetically active) cells was recorded at C/Chl *a* above 60, corresponding to the range of light inhibition of algae growth rate; moreover, an increase in the dispersion of cell sizes up to 50% at C/Chl *a* of about 100 was registered.

4. Discussion

Light dependences of algae growth rate and specific chlorophyll content reflect the nature of the work of photochemical systems and enzymatic apparatus of cells under different cultivation conditions; as a result, they are often applied in hydrobiological practice to model the dynamics of phytoplankton development (Sathyendranath et al., 2009; Baird et al., 2013; Bellacicco et al., 2016). However, duration and complexity of determining these values cause certain difficulties. An alternative approach is based on the use of methods for direct detection of cell/community stress state by express-indicators. In our study, the possibility of application of several cytometric (FDA, ratio of living cells, and CV) and fluorescent (Fv/Fm) indicators for rapid diagnosis of algae functional state under stressful growth conditions was evaluated.

As known, the assessment of algae fluorescence indicators allows revealing changes in the efficiency of the primary photochemical stages of photosynthesis (Antal et al., 2001; Arellano et al., 2005). We have established a significant correlation between Fv/Fm and C/Chl *a* for three diatom species. The resulting equation allows to estimate the specific chlorophyll *a* content in the cell, normalized to biomass, taking into account the light-dependent change in pigment concentration. The coefficient of maximum quantum efficiency of photosynthesis (Fv/Fm) is widely applied in hydrobiological practice to assess algae functional indicators, in particular growth rate, depending on the availability of mineral nutrition, when exposed to pollutants of various nature (Kolber et al., 1988; Clarke et al., 1995; Garrido et al., 2013; Kulk et al., 2013). At the same time, the results of our study showed that Fv/Fm is not effective for diagnosing changes in growth characteristics of algae, cultivated under different light conditions, since the light-dependent decrease in Fv/Fm does not correlate with changes in algae growth rate and is manifested at lower irradiance. This is probably due to the fact that the irradiance level above the value, which saturates growth and photosynthesis, can be considered as excessive one (adaptive decrease in specific chlorophyll content) and then inhibiting one, causing intense photo-oxidation and destruction of both light-absorbing pigments and PS II reaction centers (Kok et al., 1956; Powles, 1984). As can be clearly seen, this is reflected in a pronounced decrease in the efficiency of the primary photochemical stages of photosynthesis with increasing light intensity, while the value of growth rate remains unchanged. According to (Konyukhov, 2009), the decrease in the quantum efficiency is due to a decrease in the number of PS II reaction centers, as well as an increase in the nonphotochemical fluorescence quenching.

Changes in the functioning of microorganisms' intracellular esterases are widely applied as indicators of the physiological state of the studied object (Dorsey et al., 1989). Thus, they are of great importance in microalgae cells for a phospholipid turnover in membranes, which is associated with metabolic activity and viability of algae cells (Dorsey et al., 1989; Garvey et al., 2007). We applied the change in FDA fluorescence after staining the cells with a vital marker of esterase activity – fluorescein diacetate – as a probable indicator of algae viability in a wide light range. The results of the study showed as follows: high light intensity values, leading to a decrease in algae growth rate, as well as an increase in C/Chl *a*, ratio of dead cells, and cell size variability in algae cultures, had little effect on FDA fluorescence, although the effect of extremely high irradiance was observed. Thus, *P. tricornutum* and *Nitzschia* sp. cultivation at 1200 $mkEm^{-2}s^{-1}$ led to a decrease in intracellular esterase specific activity by about 25%–30%. The greatest drop in FDA fluorescence (by 80%) was recorded for *S. costatum*, cultivated at 530 $mkEm^{-2}s^{-1}$, and was associated with irreversible cell destruction and algae death. As can be seen, FDA fluorescence is resistant to external factors, if the restriction of algae growth is reversible, and the level of an affecting factor does not lead to irreversible cell destruction and death of the studied object. This is confirmed by the fact as follows: after *P.*

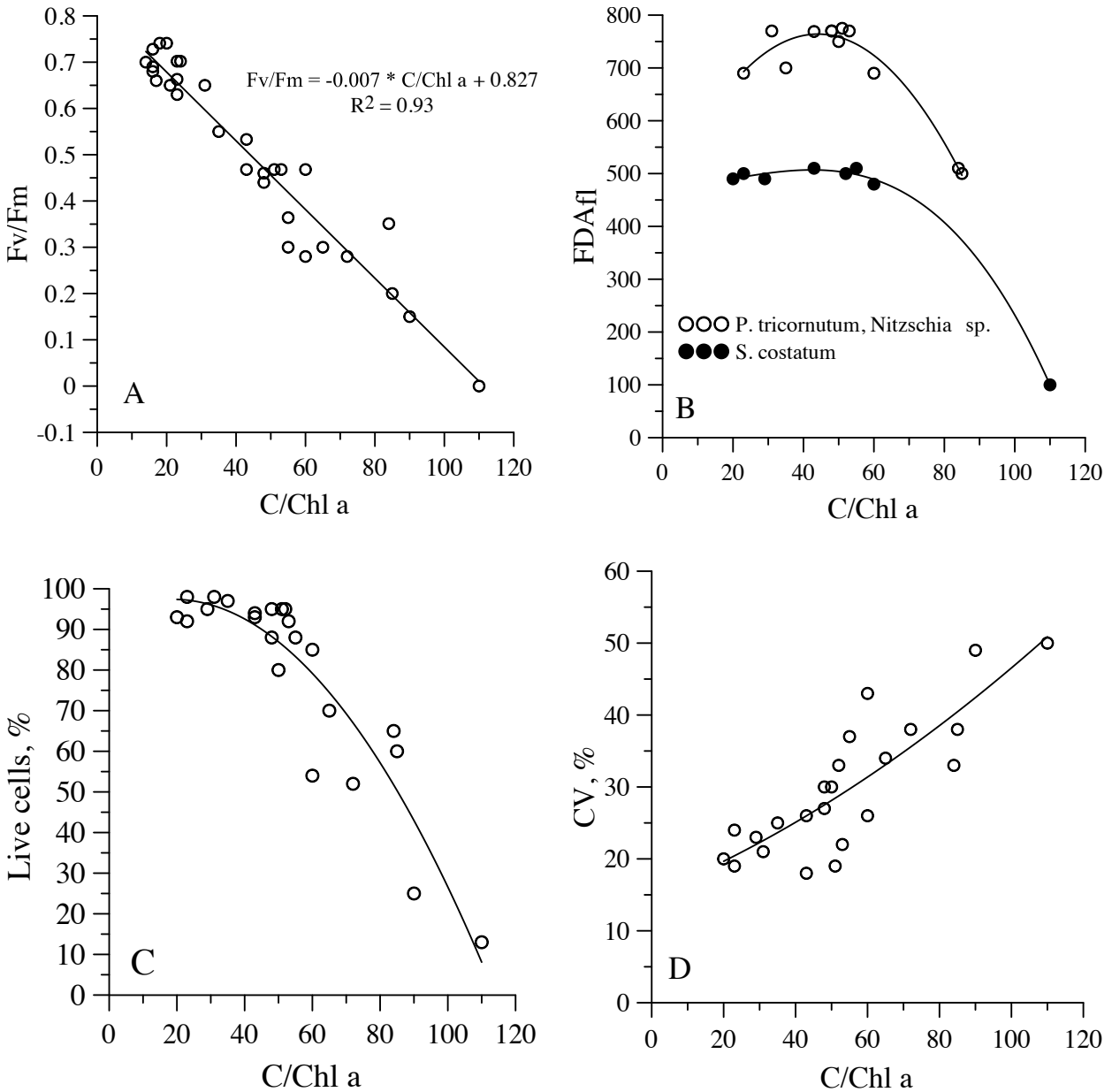

Figure 5. Correlations between intracellular C/Chl *a* and Fv/Fm (A), FDA fluorescence (B), ratio of living cells (C), and coefficient of cell size variability (D).

tricornutum and *Nitzschia* sp. transferring to optimal light conditions, algae structural and functional indicators were restored to the values, corresponding to new light conditions of cultivation. At the same time, *S. costatum* cultivation under high irradiance led to cell gradual destruction, elimination, and subsequently to death of the culture. Thus, a degree of algae survival and a possibility of restoring functional activity under light inhibition are determined not only by magnitude and duration of the stress effect, but also by species peculiarities.

In the literature, there are practically no studies, estimating algae functional state by FDA fluorescence. Only fragmentary data can be found, which describe changes in algae intracellular esterase activity, resulting from exposure to various toxic substances. The results, given in that studies, also showed that FDA fluorescence does not correlate with functional indicators of algae cells, that are capable of restoring their characteristics in case of changes in environmental conditions towards values optimal for algae growth (Franqueira et al., 1999; Prado et

al, 2009, 2011; Solomonova and Akimov, 2013; Prado et al., 2015). This once again confirms the proposed hypothesis about the feasibility of using FDA fluorescence method to assess a lethal effect of external factors on algae structural and functional characteristics.

The ratio of living, inactive, and dead cells in the suspension can also be applied to evaluate the viability of the population, since a significant decrease in the value will indicate a negative and probably irreversible effect of the factor on algae functional state. For *P. tricornutum* and *Nitzschia* sp., insignificant accumulation of dead and damaged cells (ratio of 25%–30%) was registered under inhibitory irradiance of 1200 $mkEm^{-2}s^{-1}$; it provided a complete restoration of algae functional activity, when they were transferred to favorable light conditions. At the same time, for *S. costatum*, content of dead and inactive cells in the culture reached 85% at inhibitory irradiance of 530 $mkEm^{-2}s^{-1}$; after algae transferring to optimal irradiance, their functional state was not restored, and subsequently algae died. It should be noted as follows: an increase in the ratio of inactive and dead cells in culture correlates with an increase in cell size variability (CV). A sharp increase in CV for *S. costatum* population under inhibitory irradiance is due to the appearance of single, large, and deformed cells, which is confirmed by direct observation under a light microscope.

In total, the results showed that under optimal light conditions, the ratio of living cells in a population of every studied species was at least 85%, and CV values were below 30%. In turn, a decrease in algae growth rate and an increase in the intracellular carbon to chlorophyll *a* ratio under the adverse effect of an external factor (in this case, high irradiance), is manifested in a decrease in the ratio of living cells in a population and an increase in their cell size variability (more than 30%). We assume that such ranges of variability of the analyzed indicators (ratio of living cells and CV) will also be observed under the effect of other factors, reflecting the degree of their effect on algae structural and functional indicators.

5. Conclusion

The markers of algae functional state, used in this work, can be divided into indices, indicating a decrease in cell viability, which results from "aging" of the algae population or adverse effects of environmental factors, and indices, allowing to assess a lethal effect of a stress factor on microalgae cells. Such indicators, as ratio of intracellular components (C/Chl), cell sizes dispersion (CV), and relative variable chlorophyll *a* fluorescence, are highly sensitive to factors, that inhibit algae growth; they quickly restore under favorable conditions, if there is no irreversible cell destruction. A fundamentally different character of changes was observed, when measuring the activity of esterase group enzymes (FDA fluorescence). The light intensities, leading to a decrease in growth rate and relative variable chlorophyll *a* fluorescence, as well as an increase in C/Chl *a* and CV of cells, had little effect on the activity of these enzymes under light conditions, where algae viability was preserved. We assume as follows: FDA specific fluorescence and the number of viable algae cells are more conservative indicators, which allows applying them not only for assessing the "current" state of marine biocenoses, but also for prognostic purposes and environmental modeling.

FDA_{fl}, Fv/Fm, and the ratio of living and dead cells in a population can be applied for an operational integral assessment of marine ecosystem state and functioning in connection with abiotic factors, anthropogenic load, and control of the sanitary and biological state of natural waters. This approach makes it possible to avoid the use of laborious traditional evaluation methods and to improve quality and efficiency of diagnosing stress conditions in a phytoplankton community.

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