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Structural and Functional Characteristics of the Phytoplankton Community in Coastal Waters of the Black Sea

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Abstract—The results of a study of the structural and functional characteristics of phytoplankton in the coastal region of the Black Sea using flow cytometry are presented. The data on the seasonal variability of the biomass of three algal groups (Synechococcus, pico-eukaryotic phytoplankton, and nanophytoplankton), chlorophyll *a* content, percentage of living cells, and FDA (diacetate fluorescein) fluorescence characterizing the functional state of algae are obtained. A significantly positive relationship is found between the values (biomass and autofluorescence of chlorophyll) determined on the flow cytometer and the total content of chlorophyll *a*, calculated using standard methods. The effect of temperature, illumination, and content of nutrients in water on the biomass and the FDA fluorescence of three isolated groups of algae is shown. The nitrate content and temperature have no significant effect on the abundance of pico and nanophytoplankton, while a reliable relationship is established between the biomass of nanophytoplankton and the concentrations of dissolved forms of mineral phosphorus. An inverse statistically significant correlation is found between the light intensity and the biomass of picoeukaryotic phytoplankton. It is demonstrated that the abiotic environmental factors considered in the study do not significantly affect the FDA fluorescence value, except for temperature: in the warm period of the year, the picophytoplankton are most active in the Black Sea, while the cold period of the year is favorable for the development of nanophytoplankton.

Keywords: flow cytometry, *Synechococcus*, picoeukaryotic phytoplankton, nanophytoplankton, biomass, environmental factors, fluorescein diacetate, chlorophyll *a*, Black Sea

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INTRODUCTION

Phytoplankton, as the basic component of aquatic ecosystems, determines their functional status and productivity and makes a crucial contribution to the synthesis of organic matter in water bodies. The main parameters characterizing the functional status of marine microalgae traditionally include growth and photosynthetic parameters and also concentrations and relationships of the main intracellular components (C/Chl, C/N). Determination of these parameters requires, as a rule, long and labor-intensive measuring procedures and, therefore, cannot be express indicators in case of routine field studies. In addition, it is difficult to monitor rapid effects of the environmental factors using these methods.

As modern research methods have been developed, in particular, the method of flow cytometry in combination with vital stains (Berglund and Eversman, 1988; Davey and Kell, 1996; Jochem, 1999), it becomes possible to use the fluorescent characteristics of algae for estimating the functional status of phytoplankton. It is suggested to use the value of fluorescence of cells stained with a vital fluorescein diacetate (FDA) as such an integral parameter of the functional status of algae; this value characterizes the total metabolic activity of the cell suspension, as the FDA composition includes the substrate specific to enzymes of the esterase group and allows recognizing live and dead cells of microalgae based on their membrane permeability (Dorsey et al., 1989). If the ratio of living to dead cells of algae is actively used in laboratory and industrial cultivation (Bentley-Mowat, 1982; Gilbert et al., 1992; Davey and Kell, 1996; Jochem, 1999), the application of the specific FDA fluorescence as an indicator of the metabolic activity of the microalgae communities has not been reflected in the studies on marine phytocenoses. Such approach is relatively novel in the practice of hydrobiological studies, but is rapid and characterized by easy measurements and possibility of automatization.

An analysis of spatial and temporal variability of pico- and nanoplankton, which has been poorly studied because of their size characteristics, is also of interest in the study of phytoplankton functioning in the Black Sea. The data on the distribution of these groups of microorganisms are few and fragmentary (Zaika et al., 1989, 1991; Shalapenok, L.S. and Shalapenok, A.A.,

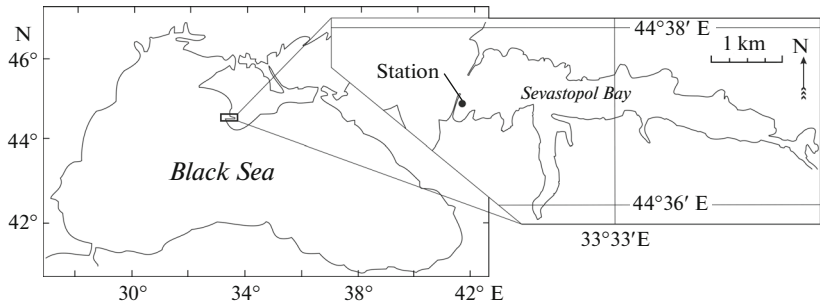

Fig. 1. Location of the sampling station in the coastal waters of the Black Sea in the area of Sevastopol.

1997; Uysal, 2000, 2001) and are certainly insufficient for understanding their role in the ecosystem.

The aim of this work is to study the seasonal dynamics of the biomass and fluorescent parameters of pico- and nanoplankton in the coastal waters of the Black Sea using flow cytometry.

MATERIALS AND METHODS

The study was conducted at the base of the Department of Ecological Physiology of Algae and Department of Plankton, Kovalevsky Institute of Marine Biological Research. The samples were collected in the surface layer (0.1 m) monthly from January to December 2014 at the model station at a depth of 5 m in Sevastopol Bay on the inner side of the southern protective pier approximately 300 m from the entrance to the bay (Fig. 1). The pier slows down the water exchange rate and protects Sevastopol Bay from the wave action. A mussel plantation is located no less than 60 m from the sampling station close to the entrance to the bay (Finenko et al., 2017). A total of 120 samples were collected. The data on the content of nutrients in the Black Sea obtained by a researcher from the Department of Aquaculture and Marine Pharmacology during that period were used in this study. Hydrochemical parameters were determined according to (Oradovskii, 1993): mineral phosphorus was measured according to Murphy–Riley and ammonium according to Grosshof–Johansen, nitrates were determined by reduction to nitrites with copper-plated cadmium and their subsequent determination using a colored reagent, and silica was determined using the silicon molybdenum blue complex.

The chlorophyll concentration was determined by a common spectrophotometric method (Jeffrey and Humphrey, 1975).

Water temperature was measured at the moment of sampling. Illumination was measured during the daylight hours every hour using a U-116 luxmeter. A transitional coefficient from illumination displayed in lux to the intensity of solar radiation (PAR) was accepted to be equal to 1000 lx = 20 $\mu E/m^2$ s (Parsons et al., 1982).

Flow cyrometrical analysis. The dynamics of the abundance of pico- (cell size of 0.2–2 μm) and nanophytoplankton (cell size of 2–20 μm) was studied using a Cytomics^{TM} FC 500 flow cytometer (Beckman Coulter, United States) equipped with a single 488-nm argon-ion laser and CXP software. Only indicated groups of algae were studied because the flow cytometer could not sieve cells above 35 μm. The total abundance of the microalgae groups was determined in unstained samples by gating the cell populations on two-parameter cytograms of the direct light scattering (FS) and autofluorescence in the red (FL4, 675 nm) and orange (FL2, 575 nm) spectral regions on dimensionless logarithmic scales (Mukhanov et al., 2016).

Orange fluorescence of phycoerythrin (FL2, 575 nm) was used for identifying the clusters of *Synechococcus* picocyanobacteria (Marie et al., 2005, Mukhanov et al., 2016). These groups form clusters on the cytograms which are characterized by a high content of phycoerythrin and its absence in picoeukaryotic phytoplankton.

The abundance of live cells of microalgae was determined in samples stained with FDA fluorochrome (Molecular Probes, United States) by gating the cell populations on two-parameter cytograms of the direct light scattering (FS) and FDA fluorescence (excitation and emission maxima, 494 and 518 nm) in the green region of the spectrum (FL 1 channel, 525 nm) on dimensionless logarithmic scales. Fluorescein diacetate (FDA), which includes a substrate, is specific to enzymes of the esterase group and is a marker of the metabolic activity in live cells and the intensity of its fluorescence is proportional to the physiological activity of the studied cells (Dorsey et al., 1989; Gilbert et al., 1992).

For the flow cytometry analysis, 3-mL aliquots were collected in three replicates. Staining of pico- and nanoplankton with FDA fluorochrome was performed according to the techniques described earlier (Solomonova and Mukhanov, 2011; Solomonova, 2016) immediately after sampling. Average values of accumulated FDA fluorescence per cell were calculated, indicating it as a value of FDA fluorescence.

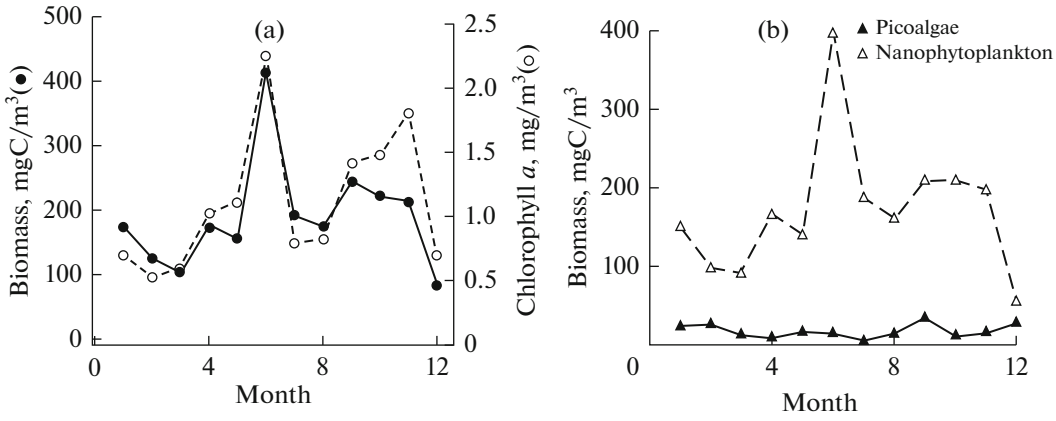

Fig. 2. Seasonal changes in the chlorophyll *a* concentration and biomass of algae (a), biomass of identified groups of algae (b).

The biomass of the studied size groups in carbon units was calculated using the coefficients determined for *Synechococcus* (Heldal et al., 2003), for picoeukaryotes (Worden et al., 2004), and nanophytoplankton (Verity et al., 1992), respectively.

The flow cytometry data were processed using Flowing software 2 (www.flowingsoftware.com).

Statistical analysis. Statistical analysis of the data was performed using Microsoft Exel 7.0, Statistica-5, Grapher-9, and Sigma Plot packages. The arithmetic mean and standard deviation (SD) for the abundance and proportion of living cells of the examined size groups (minimum of 3000 cells for each sample) were calculated in three replicates. The significance of differences between random means was estimated by the paired t-test and the coefficient of correlation (R). Regression equations were obtained by linear regression analysis (P—95%).

RESULTS

The minimum values of the chlorophyll *a* concentration and the total biomass of pico- and nanophytoplankton were recorded in winter and early spring periods, 0.5–0.7 mg/m^3 and 100–200 mgC/m^3, respectively. The parameters increased simultaneously during the spring period up to June at temperature 9–18°C (approximately 2.5 times). In July, as water warmed up and insolation increased, the concentration of chlorophyll *a* in algae decreased on average 1.4 times and remained within 0.9–1.5 mg/m^3. In October and November, when the temperature dropped and stratification broke down, the concentration of chlorophyll *a* increased to 1.5–1.7 mg/m^3; the algal biomass insignificantly increased and averaged 220 mgC/m^3. The maximum values of the phytoplankton biomass were recorded in June (450 mgC/m^3) and exceeded 2.5 times its values observed in winter (Fig. 2a). It should be noted that the algal biomass was measured using a flow cytometer and the chlorophyll *a* concentration by the standard spectrophotometric method (Jeffrey and Humphrey, 1975), where cells of larger than 35 μm were counted. This probably explains the higher values of chlorophyll *a* when compared with the biomass in autumn when, according to (Finenko et al., 2017), large fractions and filamentous colonies of algae dominated in phytoplankton.

The linear correlation (R^2 = 0.81) was found between values of the total autofluorescence of algal cells (FL4 channel, 675 nm) measured using a flow cytometer and the chlorophyll *a* content. A linear correlation with a coefficient of determination of $R^2 = 0.8$ was also found between the biomass of the studied algal groups and the chlorophyll *a* content (Fig. 3).

When considering the contribution of the biomass of picophytoplankton ((*Synechococcus* and eukaryotic picophytoplankton), it should be mentioned that it practically did not change during the annual cycle and was, on average, 18 mgC/m^3 (Fig. 2b). During the entire study period, this group of algae made the minimum contribution to the total phytoplankton biomass: from 8% in the warm period of the year (water temperature >15°C) to 26% in a cold period of the year (water temperature <15°C). Nanoplankton made the largest contribution to the biomass; its abundance tended to increase from winter to summer (Fig. 2b). The second peak of the nanoplankton biomass occurred in autumn.

Table 1 presents the correlation coefficients formally calculated between abiotic environmental factors and the biomass of the studied groups of algae. The interpretation of the coefficients should be made with due regard for possible causal relationships between the compared values. Thus, a low correlation between the abundance of pico- and nanoplankton and water temperature and concentrations of nitrates in the water column may be a result of poorly expressed seasonality of these parameters or considerable differences in their seasonal dynamics. A significant indirect correlation between the biomass of

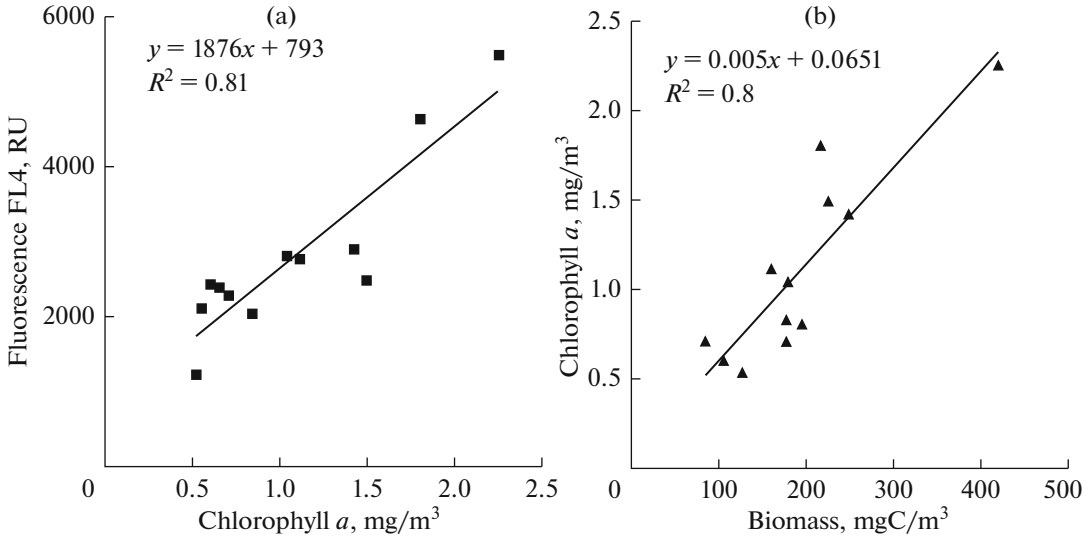

Fig. 3. Relationship between chlorophyll *a* and autofluorescence of algal cells (a), chlorophyll *a* and biomass (b).

picokaryotic algae and solar irradiance demonstrates the pattern when, in the warm period of the year, excess insolation leads to a decrease in the biomass of picokaryotic algae.

Significant positive values of r between the biomass of nanophytoplankton and the concentration of phosphorus in the marine environment were obtained. However, a more reliable interpretation of the similarity of these values may be made with due regard for the combined effect of abiotic factors by calculating the partial correlation coefficient or application of methods of multidimensional statistical analysis (e.g., canonical correlation analysis).

The percentage of live cells did not show a seasonal variability and constituted from 70 to 100% during the study period (Fig. 4). It should be noted that FDA stains not only live active cells, but inactive cells at the resting stage with undisturbed cytoplasmatic membrane as well. The fluorescence of such cells is weaker compared to live active cells.

Hence, we introduce a parameter such as the value of the mean FDA fluorescence per cell for the studied group of algae. We used this parameter earlier for the description of the functional state of algal cultures under the effect of different abiotic environmental factors (Solomonova and Akimov, 2012). The data presented in Fig. 5 demonstrate that, during the winter–spring period, the FDA fluorescence for picophytoplankton (*Synechococcus* and picokaryotic algae) had low values; this parameter for nanophytoplankton had high values at low temperature. As water warmed up in the middle of May, the FDA fluorescence values increased on average 2–3 times and practically did not change by October. When the temperature dropped in the autumn–winter period, the FDA fluorescence value decreased in picoalgae. The inverse dependence of the FDA fluorescence on temperature was obtained for nanophytoplankton (Fig. 6). However, a high percentage of live cells for all groups of algae during the study period indicates the maintenance of viability of algae despite the decrease in the intracellular esterase activity in cells.

Other abiotic factors did not have a considerable effect on the value of FDA fluorescence.

DISCUSSION

The results make it possible to confirm the hypothesis proposed in earlier studies of the absence of a considerable contribution of large cells of algae to the total chlorophyll in the coastal zone of the Black Sea. Thus,

Table 1. Coefficients of pair correlation between the studied variables*

Biomass, mgC/m^3	I, E/m^2 day	T, °C	NO_3	NH_4	PO_4	Si	Abundance, 10^3 cells/mL
Synechococcus	-0.16	0.37	–0.25	0.06	0.25	<u>0.67</u>	<u>0.97</u>
picoeukaryotic algae	<u>–0.75</u>	–0.53	0.18	0.03	–0.47	0.35	<u>0.98</u>
nanophytoplankton	0.46	0.53	–0.28	–0.4	<u>0.63</u>	0.11	<u>0.87</u>

* Statistically significant values ($p < 0.05$) are underlined.

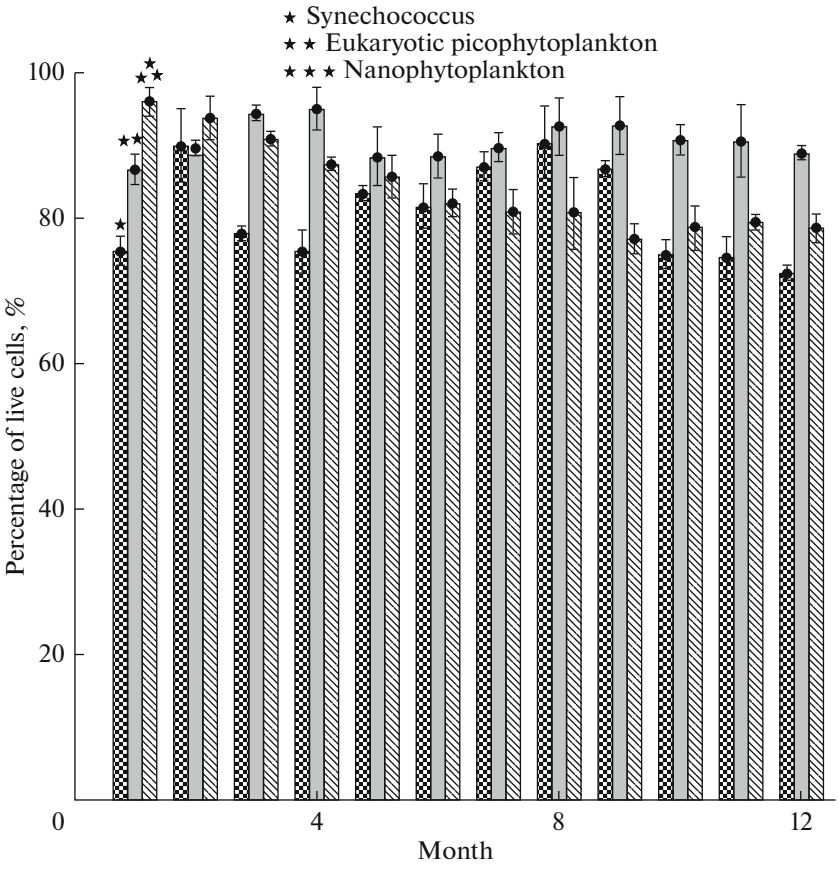

Fig. 4. Seasonal dynamics of the proportion of live cells of the studied algal groups.

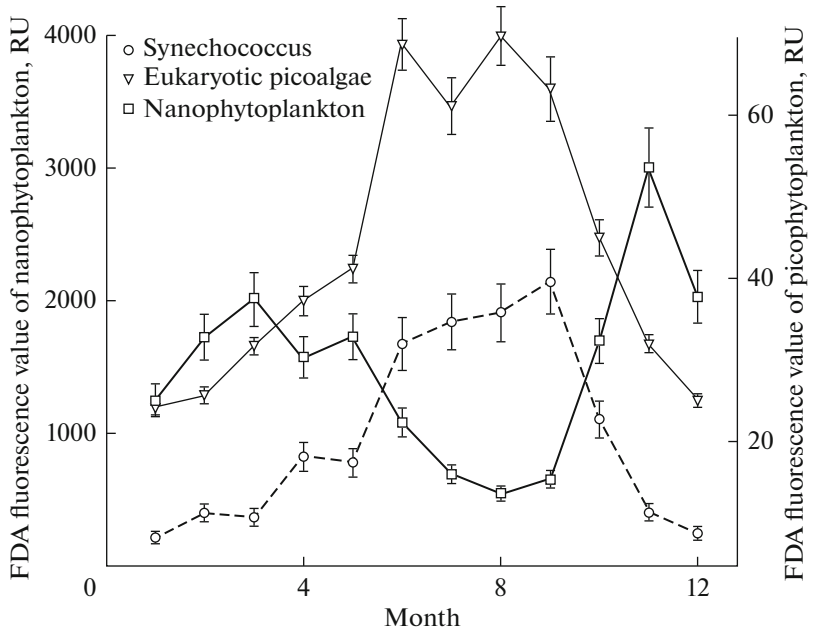

Fig. 5. Seasonal trends in FDA fluorescence values of identified algae groups.

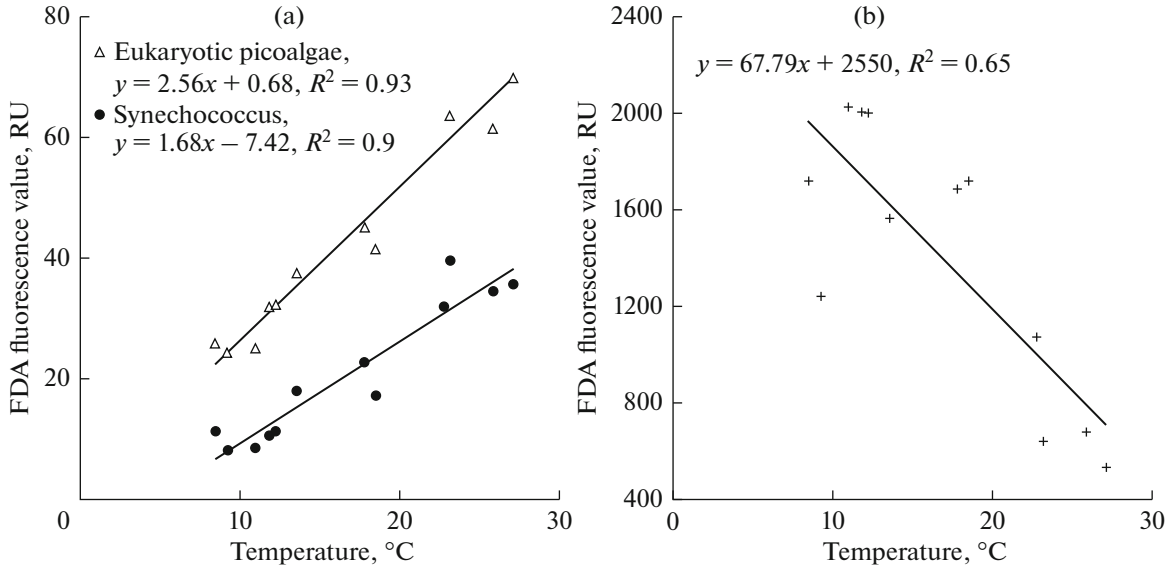

Fig. 6. Relationship between the value of FDA fluorescence and water temperature for picophytoplankton (a) and nanophytoplankton (b).

Osadchaya (2007) analyzed the seasonal size spectra of phytoplankton based on chlorophyll *a* measurements in the Black Sea and demonstrated the dominance of small phytoplankton (cells from 0.2 to 10 μm) during the largest period of the studies. The author marked a general trend of an increasing role of small cells in the phytoplankton structure in the bay, which was especially evident when considering values of total relative contributions of pico- and nanophytoplankton, which constituted from 64 to 75% of the total concentrations of chlorophyll *a* depending on the region and to 70% for the bay on a whole. This is probably explained by a significantly positive correlation between values measured using a flow cytometer and the chlorophyll *a* concentration measured by the spectrophotometric method.

Ranges of changes in the quantitative parameters of picophytoplankton obtained for the Black Sea coastal waters generally corresponded to the earlier published data for the Black Sea (Stel'makh, 1988; Osadchaya, 2007; Mukhanov et al., 2016). Thus, Stel'makh (1988) demonstrated that the proportion of picophytoplankton in Sevastopol Bay amounted from 18 to 44% of the primary production and from 20 to 40% of chlorophyll *a* during the year. Seasonal changes in the biomass of nanophytoplankton mainly formed by the coccolithophore *Emiliania huxleyi* and small diatom species (*Skeletonema costatum,* algae of the genus *Chaetoceros*) also corresponded to the earlier results (Stel'makh et al., Finenko et al., 2011).

It has been often reported in the literature that low temperature and light intensity in winter provide advantages for the development of picoeukaryotic algae, whereas high temperature and light intensity are favorable for cyanobacteria (Partensky et al., 1999). Shapiro (1990) put forward the main hypotheses indicating the specific features of cyanobacteria that allow them to compete with picoeukaryotic algae and contribute to phytocenosis development: (a) cyanobacteria are able to develop at high water temperature, (b) cyanobacteria survive in a wide range of light intensity, and (c) cyanobacteria blooms develop in waters with low concentrations of nutrients. The possibility of *Synechococcus* to grow in a wide range of light intensity upon a limitation of algae by nutrients in water that forms its development in the summer and early autumn periods is provided by the phycoerythrin pigment, which they use as the inner source of nitrogen (Wyman, 1992) and for adaptation to different light conditions (Glover et al., 1987; Takahashi et al., 1989).

We observed that temperature and nitrate concentrations in the water column did not have a great influence on the abundance of picoplankton, which was confirmed in the work (Worden et al., 2004), including that for the surveyed region (Mukhanov et al., 2016). However, most researchers support the theory that temperature is one of the crucial factors controlling the abundance of picoalgae (Pick and Caron, 1987; Simon et al., 1999, Agawin et al., 2000). As concerns nano- and microphytoplankton, it was repeatedly reported in the literature that temperature is not the main factor determining the value of the primary production in the World Ocean (Burke et al., 1997). The maps of distribution of water productivity in the World Ocean demonstrate that the value of the primary production depends mainly on the hydrological processes of various scales causing the enrichment of surface waters with nutrients (Koblets-Mishke, 1985).

A large complex of factors affects the seasonal variability of the nanophytoplankton biomass in the water

column. The physical factors include light intensity, temperature, and the role of physical processes in the formation of the chemical structure of water in the study region. Small species of diatoms, flagellates, and the coccolithphore *E. huxleyi* make a major contribution to the nanophytoplankton biomass in the Black Sea. According to the data (Finenko et al., 2017), the mass development of the coccolithphore *E. huxleyi* (cell diameter of 6–11 μm) in the phytoplankton community occurred from April to August and in autumn 2014. During this period of the year, we obtained the maximum biomasses of nanophytoplankton using a flow cytometer. Water bloom as the result of the peak development of *E. huxleyi* was caused by vertical water circulation and the input of a great amount of mineral phosphorus to the euphonic zone. Mikaelyan et al. (2011) demonstrated in their studies that the concentration of phosphates in the environment is the most important factor for *E. huxleyi* growth, which is confirmed by the statistically significant correlation between the biomass of nanophytoplankton, which is mainly formed in our opinion by *E. huxleyi* and concentrations of these nutrients.

The differentiating criteria for recognizing live and dead cells may be any features of living things such as the ability of the cell to divide and form colonies, mobility, manifestation of the metabolic activity, enzymatic reactions, the state of the permeability barrier, ATP accumulation, morphological characteristics, etc. Fluorescein diacetate (FDA) is widely used to mark live cells in the studies on microalgae cultures and natural phytoplankton (Dorsey et al., 1989; Gilbert et al., 1992). In the study area, the percentage of live cells of pico- and nanophytoplankton did not show seasonal variability and averaged 80% during the year. However, it should be taken into account that ffluorescein diacetate stains not only live active cells, but live inactive cells at the resting stage as well. Sicko-Goad et al. (1989) demonstrated in their studies that, during the year, inactive cells are present in the phytoplankton community along with live active cells, which do not have morphological modifications and remain viable; thus, they characterized such cells as physiologically resting cells. Latour et al. (2004) noted that, despite low water temperatures in winter, benthic cyanobacteria had a low level of esterase activity which, in their opinion, was equivalent to the resting state of algae Such a resting state is characteristic of many species of algae from different taxonomic groups (McQuoid et al., 2004; Wang et al., 2013). Therefore, it is appropriate to use the average FDA fluorescence per cell in the study on the functional status of natural communities of microalgae. It is shown in our study that the value of the FDA fluorescence per cell has a significant positive correlation with the water temperature in the Black Sea for picophytoplankton and a negative correlation for nanophytoplankton. Other environmental factors did not have a considerable effect on the seasonal dynamics of the FDA fluorescence value. The maximum values of the FDA fluorescence of picophytoplankton in summer may be consistent with the suggestion proposed by Agawin and Duarte (1998) that the rate of cell division of this size group is higher than in the other period of the year, which may result in the maximum biomass (35 mgC/m^3) obtained in September. In a cold period of the year, nanophytoplankton is more active under increased concentrations of nutrients in the coastal waters of the Black Sea; its main representatives are small diatoms, in particular *S. costatum* and algae of the genus *Chaetoceros* and the coccolithophore *E. huxleyi* (Stel'makh et al., 2009), which is confirmed by our results demonstrating high values of the esterase activity in cells of nanophytoplankton.

CONCLUSIONS

The study of seasonal variability of the biomass, chlorophyll *a* content, accumulated FDA fluorescence (activity of intracellular esterases), and percentage of live cells of three groups of microalgae (*Synechococcus*, picoeukaryotic algae, and nanophytoplankton) were conducted at the model station in the Black Sea area.

The seasonal dynamics of the biomass of pico- and nanophytoplankton and chlorophyll *a* content was characterized by high values in autumn and the maximum in June. The contribution of picophytoplankton (*Synechococcus* and eukaryotic picoalgae) to the total biomass averaged 20%.

A statistically significant correlation was found between the chlorophyll *a* content measured by a standard spectrophotometric method and the total autofluorescence of chlorophyll and biomass measured using a flow cytometer.

It is shown that the percentage of live cells did not show seasonal variability and was on average 80% during the year. Values of the FDA fluorescence per cell had a positive correlation with the water temperature in the Black Sea for *Synechococcus* and picoeukaryotic algae and a negative correlation for nanophytoplankton, which confirms once more the fact that the cold period of the year is characterized by the activity and development of nano- and microphytoplankton, whereas in the warm period of the year picoalgae are able to compete and their blooms occur in the phytocenosis.

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