Physiological heterogeneity of an algal population: classification of *Scenedesmus quadricauda* cenobia by the features of their photosynthetic apparatus

OCEANOLOGIA, 39 (2), 1997.
pp. 163–175.

KEYWORDS
Chlorophyll
Fluorescence
Induction curves
cluster analysis

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Manuscript received April 25, 1997, in final form June 12, 1997.

Abstract

An analysis of the typological composition of individual cenobia of the microalga *Scenedescemus quadricauda* using typification of chlorophyll fluorescence induction curves is presented. Cluster analysis was applied to separate various types of induction curves. The 12 representative types of fluorescence induction curves of *S. quadricauda* are given. The possibility of division into separate clusters confirms the assumption of the discrete states of the photosynthetic apparatus. The connection between the functional structure of the population and its growth stage can be established.

1. Introduction

Many literature studies deal with the responses of microalgal populations to environmental pressure. As a rule, in order to characterise the physiological state of algae, researchers have concentrated on changes in cell numbers, or photosynthetic rates (measured by oxygen evolution or carbon dioxide assimilation) or, on changes in various cell components, such as proteins, photosynthetic pigments and accumulated metabolites. However, such an approach does not enable one to consider the non-uniform response of individual algae cells, which seems to be an important property of a biological population. Although the reactions of individual organisms in a population are known to be heterogeneous, investigations of the problem in algae have so far been limited by methodological difficulties. We assumed that the nature of algae distribution according to certain important functional parameters reflects the physiological state of the population at a given instant and that a definite functional structure of the organisms corresponds to each state of the population. This implies that a knowledge of a population's functional structure permits the changes in and the dynamics of population development to be predicted.

One of the most important parameters of the functional activity of autotrophic cells are those of photosynthesis, the principal process providing a green cell with energy. Previously, using microfluorometric measurements, we estimated the changes in *Chlorella* and *Ankistrodesmus* cell distribution according to chlorophyll content and the efficiency of primary photosynthetic reactions during the growth of cultures affected by toxicants or under conditions of nitrogen deficiency (Pogosyan *et al.*, 1991).

It is well known that a number of important photosynthetic reactions affect the fluorescence induction curve of chlorophyll *a* recorded in samples previously adapted to darkness. The curve is measured after the actinic light has been switched on (Bradbury and Baker, 1984; Clayton, 1980). The microfluorometric device developed in our laboratory allows the fluorescence induction of a single cell and even of an individual chloroplast to be measured, which makes it possible to evaluate the functional state of the photosynthetic apparatus. Thus, it seems quite possible to describe an algal population structure in terms of its major energetic characters. In our opinion, this could be done by comparing the fluorescence induction curves of the individual organisms in the different states of a growing population.

The fluorescence induction curve of chlorophyll *a* is produced by the overlapping of the elementary curves due to numerous possible, differentiated chloroplast membranes. At the same time, the fluorescence induction curve represents an 'image' of a given organism. In general the fluorescence induction curve of plant cells can be analysed in different

ways. To analyse the typological composition of individual *Scenedescemus quadricauda* cenobia, we have developed in this paper a method for typifying chlorophyll induction curves. Such methods of assessing the functional state of a population could be useful in ecological studies.

In the general case this typification assumes a certain discreteness of the conditions under which it is carried out. At present the existence of discrete photosynthetic membrane states and their trigger-switching mode of action are known from the literature (Gilyarov, 1990; Shvarts, 1980). This has been taken into account in the present paper.

2. Materials and methods

The experiments were conducted on a culture of the green algae *S. quadricauda* obtained from the collection of the Department of Hydrobiology, Faculty of Biology, Moscow State University. The algae were cultivated for up to 52 days in 10% Tamiya's medium (Tamiya *et al.*, 1953) in 300 ml flasks at 26–27°C under a fluorescent lamp illumination of 8 W m^{-2}. The number of cenobia was determined by counting them in a Nagott chamber (Fedorov, 1979). Only cenobia containing four cells were counted and used in the fluorometric measurements.

The measurements of chlorophyll fluorescence induction curves was conducted with a Lumam–I3 luminescence microscope (LOMO, Leningrad), equipped with an FMEL 1 fluorescent attachment. This recorded the luminescence from the sample at a resolution of *ca* 0.1 s. The excitation fluorescence (at an intensity of 10 W m^{-2} at the cell surface) was provided by a KGM 9–70 halogen lamp and passed through water and glass (SZSC–22) filters. With the objective set at '40x', the diameter of the area from which the fluorescence was collected was 37.5 μm. *S. quadricauda* cells were accommodated to the dark for 15–20 min. before the actinic light was switched on. In each experiment the cenobia for assay was selected at random. The fluorescence induction curves were recorded for no less than 200 s.

In each experiment (from day 4 to day 52 with 2–3 day intervals) the fluorescence induction kinetics were measured from 30–50 cenobia (a total of 846).

3. The mathematical treatment of the data

The intensity of fluorescence (I), measured at the time 0, 10, 25, 50, 75, 100, 125, 150, 175, and 200 s, was chosen as an initial feature on which the form of the induction curve was evaluated. Preliminary analysis of the data has led to the conclusion that the most characteristic features of various types of induction curves are the differences in the fluorescence amplitudes

at consecutive instants. In this connection, we analysed the behaviour of the following 9 differences in the fluorescence intensity logarithms:

$$\begin{aligned} \Delta_1 &= \ln I_{10} - \ln I_0, \\ \Delta_2 &= \ln I_{25} - \ln I_{10}, \text{ and } etc. \end{aligned} \tag{1}$$

The various types of induction curves were separated using a type of cluster analysis known as 'the K-means method' (Duran and Odel, 1977). The advantage of this method, in comparison with well-known hierarchical schemes, consists in the fact that in the former case, the matrix of distances between the objects does not need to be calculated. Therefore the problems of choosing a metric space and of the means of association do not arise, neither are there any limitations to the number of objects studied. The latter circumstance was decisive for our task, as the initial set of data contained the descriptions of 846 induction curves. In addition, the maximum number of objects that can be classified using the accessible programs of hierarchical analysis does not exceed 100.

Cluster analysis (as well as other methods of multi-dimensional analysis) is usually most effective when the intervals of the changes in all features have been normalised or adjusted. The so-called standardisation of variables is very often used for this purpose. In this work we applied a linear transformation of scales of all features (*e.g.* Δ_1, Δ_2, ... and Δ_9) that is basically similar to standardisation, but in practice more convenient. For each feature both minimum ($\Delta_{\min}$) and maximum ($\Delta_{\max}$) values were determined and the nearest integer to them or rounded-off values were found. The latter were denoted as Δ_{i0} and Δ_{i1} respectively. Then the linear transformation is

$$\Delta_i = \frac{\Delta_i - \Delta_0}{\Delta_{i1} - \Delta_{i0}}. \tag{2}$$

This means that the origin of the coordinates for the i-th case is situated to the left of $\Delta_{i\,\min}$ and that $\Delta_{i\,\max}$ on this scale is close to (but less than) 1. This operation is actually analogous to the algorithm for constructing two-dimensional graphs in the automatic scaling regime.

The maximum and minimum differences between logarithms found for the initial set of data (846 induction curves) are given in Tab. 1. The formulas for the transformations of the initial data into new variables were obtained on the basis of these values.

The result of using the cluster analysis algorithm is a series of tables which not only group the whole set of objects into clusters, but also provide additional information about the quality of the clustering. In particular, the proximity of the objects belonging to one and the same cluster was demonstrated by their Euclidean distances to the centre of the cluster, standard deviation and dispersion.

Table 1. The formulas used for primary transformation of the initial data

	$\Delta_{\min}$	$\Delta_{\max}$	
Δ_1	1.729	0.000	$x_1 = (\Delta_1 + 1.7)/1.7$
Δ_2	0.860	0.475	$x_2 = (\Delta_2 + 0.9)/1.3$
Δ_3	0.636	0.380	$x_3 = (\Delta_3 + 0.6)/1.3$
Δ_4	0.353	0.597	$x_4 = (\Delta_4 + 0.4)/1$
Δ_5	0.143	0.543	$x_5 = (\Delta_5 + 0.1)/0.6$
Δ_6	0.133	0.369	$x_6 = (\Delta_6 + 0.1)/0.5$
Δ_7	0.287	0.269	$x_7 = (\Delta_7 + 0.3)/0.6$
Δ_8	0.287	0.211	$x_8 = (\Delta_8 + 0.3)/0.5$
Δ_9	0.154	0.223	$x_9 = (\Delta_9 + 0.2)/0.4$

In standard K-means cluster analysis, the number of clusters is assigned first. The number of chlorophyll fluorescence induction curve types is not known *a priori*. For this reason we compared the various clustering variants to fit the number of the induction curve types found in our experiments.

So far no 'successful' criterion for choosing the clustering variant has been developed. We believe that such a criterion might be the ratio of the average Euclidean intercluster distance (L) to the average Euclidean distance between the cluster centres and their extreme elements (H). This means that the best clustering corresponds to the highest values of this ratio, and separate clusters are compact and distant from each other. It should be noted that the number of clusters must be limited, because any increase leads to a reduction in the number of elements in certain clusters up to levels where statistical approaches are not effective.

4. Results

The results of grouping the data set studied are presented in Tab. 2. As can been seen, the increase in the number of clusters leads to a rise in the L/H ratio. However, the distinction in this ratio for grouping into 12 and 18 clusters is small (less than 4%), while the number of clusters containing less than 30 elements has increased from 2 to 5. Besides, the average distances between the clusters are shorter for 12 clusters than for 18. In our opinion, these circumstances indicate that the division of the data set into 12 clusters is the most 'successful'.

The representative types of fluorescence induction curves of *S. quadricauda* cenobia obtained after separation into 12 clusters are shown in Fig. 1. All types of induction curves are quite distinct, which suggests that this method of processing primary data is correct.

Table 2. The clustering of fluorescence induction curves of *S. quadricauda* cenobia

	Number of clusters		
	7	12	18
average distance between clusters (L), [rel. un.]	0.357	0.497	0.453
average distance from the cluster centre to extreme (H), [rel. un.]	0.196	0.149	0.130
L/H ratio	1.82	3.36	3.48I
number of clusters containing fewer than 30 elements	0	2	5

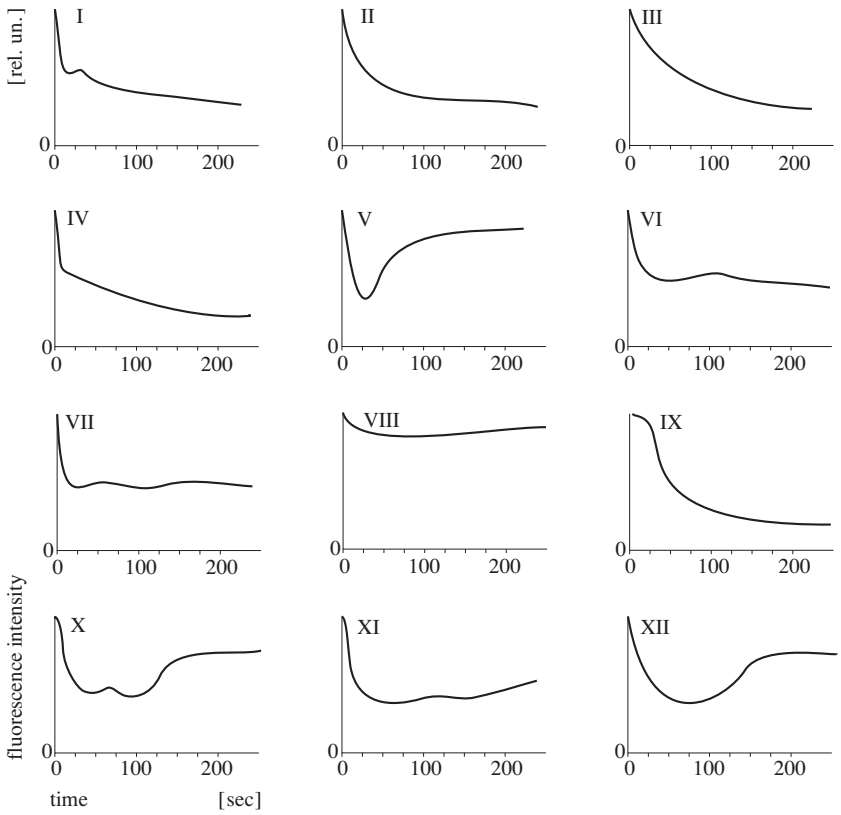

Fig. 1. Typical chlorophyll fluorescence induction curves of *S. quadricauda* cenobia, corresponding to the centres of clusters (the numbers of clusters are indicated)

The typification carried out in this study was the first step in the treatment of the data. In the second step we compared the frequencies with which various types of fluorescence induction curves occurred in cenobia of a growing *S. quadricauda* culture in which the photosynthetic apparatuses of the organisms can be in different states. By using this approach we expected to distinguish the time intervals in which the relative abundance of cells of each type was less comparable with other time intervals. In other words, this step represents second – order clustering, where the clustered objects were samples obtained from the culture at certain stages of growth, characterised by the relative abundance of cenobia of a given type in the sample.

The dynamics of changes in the numbers of 4-cell *S. quadricauda* cenobia growing in the flask culture are shown in Fig. 2. This growth curve is close to being logarithmic.

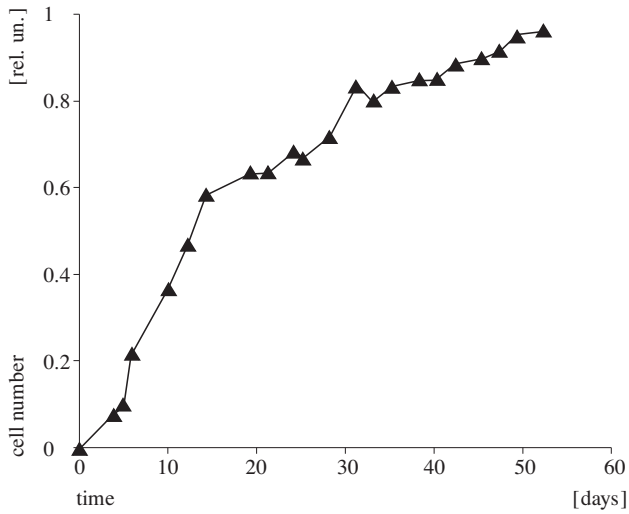

Fig. 2. The growth curve of *S. quadricauda* cenobia

For 2nd order clustering the frequencies of occurrence of different types of fluorescence induction curves on each day of the sample collection were calculated (Tab. 3). From this table it can be seen that the population of the clusters is strongly differentiated. The majority of clusters contain from 16.7 to 4.4% elements of the whole. Two clusters (III and IX) are extraordinarily small. Note that during the linear growth stage of the culture (10–33 days), induction curves of types V, X, XI and XII are practically absent. These types of curves are characteristic of the initial growth stages and of the transition to near-saturation of the culture (Fig. 2). Thus, each stage of algal development can be characterised by a given functional structure.

Table 3. Relative contents of fluorescence induction curve types of *S. quadricauda* cenobia as a function of the age of the culture

Age of culture	Types of induction curves [%]											
[days]	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
4	12	12	2	4	4	0	2	0	0	19	45	0
5	2	8	0	4	12	2	0	0	0	26	22	24
6	4	2	0	2	32	4	6	0	0	8	6	36
10	2	0	0	14	2	24	0	58	0	0	0	0
14	11	2	0	23	0	55	9	0	0	0	0	0
19	18	2	18	25	0	22	2	7	4	0	2	0
21	42	8	0	20	0	17	8	5	0	0	0	0
24	34	31	0	11	0	5	5	8	3	3	0	0
26	31	36	0	11	0	8	8	3	0	0	3	0
28	22	30	0	6	6	8	25	3	0	0	0	0
31	43	28	0	0	0	6	20	0	0	0	3	0
33	27	34	0	12	0	6	18	0	3	0	0	0
35	21	21	0	8	4	0	38	4	0	0	0	4
38	11	32	0	11	2	11	21	2	0	4	6	0
40	11	25	0	8	3	5	31	8	0	3	3	3
42	22	25	3	0	3	6	22	3	0	0	11	5
45	19	26	0	16	3	0	19	0	0	7	10	0
47	11	32	0	11	3	3	21	0	0	3	8	8
49	18	11	0	7	11	4	26	4	0	0	15	4
52	31	17	3	11	0	9	11	0	0	6	9	3
Σ	16.5	16.7	1.4	11.2	4.5	12.6	11.6	8.8	0.4	4.4	7.0	4.9

The cluster analysis of the data in Tab. 3 was performed using Manhattan metric (MHM) as a similarity measure. MHM is defined as $\Sigma(p_{ik} - p_{jk})$, where p_{ik} and p_{jk} are the relative abundance of K-type objects on days i and j respectively (Pesenko, 1982; Whittaker, 1965).

In ecological practice this measure is well-known as a 'percentage difference' (Pesenko, 1982; Whittaker, 1965) and is widely used for evaluating the similarity of the species composition of communities. Because an analogy exists between the taxonomic composition of a community and the topological composition of a population, we believe that the choice of MHM is quite possible.

The result of the MHM-clustering was obtained by nonmetric scaling in 2-dimensional space (Fig. 3). The distances between the points on this diagram were calculated on the basis of linear correlation with MHM distances between the corresponding samples. In Fig. 3 the age of the

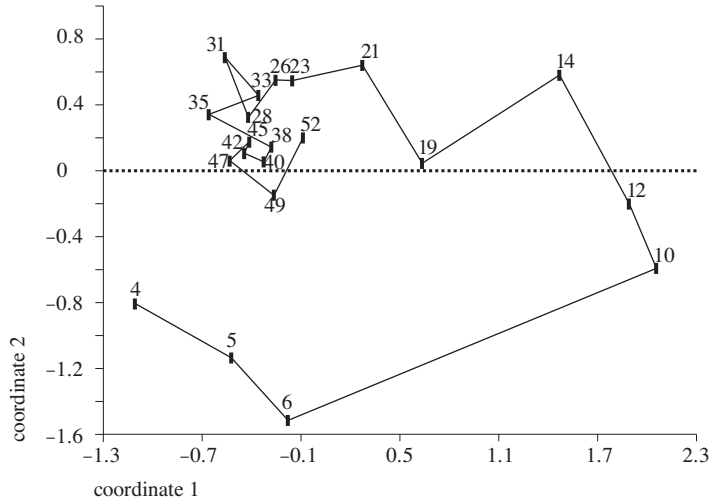

Fig. 3. The 2-dimensional presentation of 12-metric image of 'the trajectory of movement' of a *S. quadricauda* population

culture is indicated by numbers. The polygonal line linking the points in accordance with the growth of the microalgae forms a sort of 'trajectory of the population' in the space of integrated variables.

5. Discussion

Heterogeneity is one of main properties of a biological population. The individual organisms forming a population are not identical in their sizes, rates of growth, or in their response to the environmental changes. The fluorescence induction curve of a green plant recorded on switching the actinic illumination on (following prior adaptation to darkness) characterises the changes in the intensity of chlorophyll *a* fluorescence in response to the action of a standard light. At present it is known that the induction curve is the result of a number of complex processes proceeding in and within photosynthetic membranes of plant cells (Bradbury and Baker, 1984; Walker, 1981, 1989). Measurement of the fluorescence induction curves is a useful and relatively straightforward method for evaluating the primary photosynthetic reactions that is free from the uncertainties related to nonhomogeneous fluorophore distribution and the reabsorption that takes place during the application of other fluorometric methods (Collins *et al.*, 1985; Li, 1990; Sosik *et al.*, 1989). The shape of the fluorescence induction curves reflects the rate of chain transport of electrons in chloroplasts, the magnitude of the transmembrane potential at a photosynthetic membrane, the energy migration between photosystems II and I, the changes in the

rate constants of energy transfer from light-harvesting complexes to reaction centres as well as the reactions of the Calvin cycle (Clayton, 1980).

However, at present it is very difficult to connect a particular phase of the induction curve with individual photosynthetic reactions. Even if significantly simplified, the induction kinetics are described by a system of 10 differential equations (Riznichenko *et al.*, 1996; Rubin *et al.*, 1987). This complexity led us to use a formal approach to describe these kinetics.

Induction curves were registered microfluorimetrically during 200 s of illumination with a time resolution of 0.1 s; this yielded 2000 measurements for each curve, a number which seems rather excessive. Having analysed the primary data, it was found that the measurements of 10 parameters already provide sufficient information to typify the fluorescence induction curve. This was confirmed by the fact that after the partition of the curve into 10 variables, the coefficients of the pair correlation in some cases were as high as 0.65. Thus, a further increase in the number of variables led to the situation where some of them appeared as dependent variables. These variables are therefore superfluous in the typification procedure.

Considering the interaction between the light and dark stages of photosynthesis, the mathematical models indicate the possibility that discrete states of the photosynthetic apparatus exist, and suggest a trigger transition from one state to another (Plusnina *et al.*, 1994; Riznichenko *et al.*, 1994). However, these models cannot be transferred from the membrane level to that of a chloroplast, cell or cenobium. To the best of our knowledge, no experimental confirmations of key switching of chloroplast and individual plant cell states were obtained. Cluster analysis thus enables one to verify the hypothesis about the existence of series of discrete conditions in the cell's photosynthetic apparatus. Where a complete set of individual objects was successfully divided, compact discrete states were assumed to exist in the photosynthetic apparatus.

The subdivision of the data set into 12 clusters allowed local groups of objects to be separated, which upholds the assumption about the discrete states of the photosynthetic apparatus as cell formations of *S. quadricauda* cenobia. The fact that in the space of these features we have found relatively large areas occupied by individual clusters, indicates that certain photosynthetic apparatus steady-state(s) can be preserved under certain conditions.

In the case studied the sum of all internal 9-dimensional volumes of the clusters was found to be less than 1% of the volume, corresponding to the range of variables. The range of values for each variable was determined under conditions when the extreme points obtained in the experiments were included in the data (Tab. 1).

The question about the possible number of chlorophyll fluorescence induction curve types is rather complicated and cannot be solved on the basis of theoretical considerations. In any case, according to visual evaluation it should be no less than 7 types. This variant of the analysis was used by Riznichenko *et al.* (1996). However, this approach does not seem to be reasonable enough, because the selection procedure of the number of types of objects determines *a priori* the number of clusters and leads to subjectivity in the cluster analysis.

The 12 clusters obtained are very different in the number of elements. The preliminary data obtained in further experiments show that clusters containing a small number of elements are much more populated when the cultivation conditions change. This indicates that the exclusion of such small clusters from consideration is inadmissible. At the same time, a new environment or cultivation conditions would probably require the introduction of additional clusters.

The relationships between the cenobium number dynamics and the relative numbers of cenobia, belonging to each of 12 fluorescence induction types, enables a connection between the functional structure of the population and its growth to be established (Fig. 2). The possibility of forecasting microalgae number dynamics using the data on typological composition requires further investigation. However, the results obtained suggest that this is perfectly feasible. The 2-dimensional visualisation of the 12-metric image of the population structure enables 'the trajectory of movement' of the population to be described in the course of its growth during cultivation (Fig. 3). This figure may be ambiguous – it is quite possible to obtain other variants of such an image. Nevertheless, it follows from the foregoing that a considerable similarity exists between a population in the lag-phase (when cells are adapting to new conditions of growth) and that same population in the stagnation phase. The transition of the culture into the linear growth stage is accompanied by considerable changes in the structure of the population.

6. Conclusions

- Each stage of algae development is characterised by its defined functional structure.
- The measurements of the fluorescence induction curves could be a useful method for evaluating primary photosynthetic reactions.

- The measurements of 10 parameters already provides sufficient information to typify a fluorescence induction curve.
- The assumption about the discrete states of the photosynthetic apparatus as cell formations of *S. quadricauda* cenobia was confirmed.

Acknowledgements

The present work was supported by the grant MKK000 from ISF. The authors are grateful to Prof. G. Yu. Riznichenko and Dr. G. V. Lebedeva for their helpful discussion of the results and comments.

References

Bradbury M., Baker N. R., 1984, *A quantitative determination of photochemical and non- photochemical quenching during the slow phase of the chlorophyll fluorescence induction curve of bean leaves*, Biochem. Biophys. Acta, 765 (3), 275–281.

Clayton R. K., 1980, *Photosynthesis: physical mechanisms and chemical patterns*, Cambridge Univ. Press, London–New York, 350 pp.

Collins D. J., Kiefer D. A., SooHoo J. B., McDermid I. S., 1985, *The role of reabsorption in the spectral distribution of phytoplankton fluorescence emission*, Deep-Sea Res., 32, 983–1003.

Duran B., Odel P., 1977, *Cluster analysis*, Statistika, Moskva, 127 pp., (in Russian).

Fedorov V. D., 1979, *Methods of studying phytoplankton and its activity*, Moscow State Univ., Moskva, 167 pp., (in Russian).

Gilyarov A. M., 1990, *Population ecology*, Moscow State Univ., Moskva, 191 pp., (in Russian).

Li W. K. W., 1990, *Bivariate and trivariate analysis in flow cytometry: phytoplankton size and fluorescence*, Limnol. Oceanogr., 35 (6), 1356–1368.

Pesenko Yu. A., 1982, *The principles of quantitative analysis in faunal studies*, Moscow State Univ., Moskva, 287 pp., (in Russian).

Plusnina T. Yu., Riznichenko G. Yu., Aksenov S. I., Chenyakov G. M., 1994, *The effect of weak electrical interaction on the trigger system of transmembrane ion transfer*, Biofizika, 39 (2), 345–350, (in Russian).

Pogosyan S. I., Lebedeva G. V., Riznichenko G. Yu., 1991, *The connections between the functional structure of a population of aquatic plants and its dynamics*, [in:] *Problems of ecological monitoring and ecosystem modelling*, Gidrometeoizdat, Leningrad, 280–297, (in Russian).

Riznichenko G. Yu., Plusnina T. Yu., Aksenov S. I., 1994, *Modelling of the effect of a weak electric field on a nonlinear transmembrane ion transfer system*, Bioelectrochem. Bioenerg., 35, 39–47.

Riznichenko G. Yu, Lebedeva G. V., Pogosyan S. I., Sivchenko M. A., Rubin A. B., 1996, *Fluorescence induction curves recorded from individual microalgae, cenobia in the process of population growth*, Photosynth. Res., 49, 151–157.

Rubin A. B., Piteva N. F., Riznichenko G. Yu., 1987, *The kinetics of biological processes*, Moscow State Univ., Moskva, 327 pp., (in Russian).

Shvarts S. S., 1980, *The ecological laws of evolution*, Nauka, Moskva, 326 pp., (in Russian).

Sosik H. M., Chisholm S. W., Olson L. R. J., 1989, *Chlorophyll fluorescence from single cells: interpretation of flow cytometric signals*, Limnol. Oceanogr., 34, 1749–1761.

Tamiya H., Shibata K., Sasa T., Iwamura T., Morimura I., 1953, *Effect of diurnally intermittent illumination on growth and some cellular characteristics of Chlorella*, Carnegie Inst. Washington, 600, 76–84.

Walker D. A., 1981, *Secondary fluorescence kinetics of spinach leaves in relation to the onset of photosynthetic carbon assimilation*, Planta, 153 (3), 73–278.

Walker D. A., 1989, *Measurements of oxygen content and chlorophyll fluorescence*, [in:] *Photosynthesis and bioproduction: methods of determination*, Mir, Moskva, 179–185, (in Russian).

Whittaker R. H., 1965, *Dominance and diversity in land plant communitties*, Science, 147, 250–260.