BULG. J. PLANT PHYSIOL., 1997, 23(1–2), 67–76

EVALUATION OF ALGAL CULTURE VIABILITY AND PHYSIOLOGICAL STATE BY FLUORESCENT MICROSCOPIC METHODS

Irina Pouneva

Acad. M. Popov Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria

Received 24 July 1997

Summary. The viability of algae belonging to divisions *Cyanophyta*, *Chlorophyta* and *Xantophyta* has been assessed by chlorophyll autofluorescence and after staining with fluorescent dyes rodamin B, neutral rot, calcofluor white and fluorescein diacetate. For this purpose the fluorescence of algal cells from fresh and old cultures was scored. It was established that chlorophyll autofluorescence could be recommended as the most convenient method for differentiating living and dead algal cells. Since in most of the algal species investigated chlorophyll autofluorescence masked cytoplasmic fluorescence, the tests based on fluorescence of the living cytoplasm (as in the case of the fluorochromatic test and CFA) proved to be inappropriate. These viability tests which are based on the fluorescence of the dead cell cytoplasm (NR and RB) appeared to be useful for vitality assessment, because chlorophyll fluorescence was lost and did not mask cytoplasmic fluorescence. The conclusion is drawn that in this kind of investigation an individual approach should be applied for each systematic section of algae.

Key words: viability of algae, chlorophyll autofluorescence, neutral rot, rodamin B, calcofluor white, fluorescein diacetate

Abbreviations: NR – neutral rot; RB – rodamin B; CFW – calcofluor white; FDA – fluorescein diacetate; FCR – fluorochromatic reaction

Introduction

The assessment of the changes in cell viability and of the underlaying process is of great significance for the control of biotechnological processes (Jones, 1987 a,b), of

pollen quality (Heslop-Harrison et al., 1984; Georgieva and Kruleva, 1994), as well as of cases of laboratory and semi-industrial microalgal cultivation. Usually cell vitality is determined after staining of different compounds, presented in the normal cells with specific dyes (NR, RB, CFW, acridine orange). Enzyme tests could also be applied in assessing metabolic activity in viable cells (reduction of tetrazolim), as well as the integrity of the plasmalemma (Heslop-Harrison et al., 1984; Fisher et al., 1985; Coleman and Vestal, 1987; Saga et al., 1987). Induction of chlorophyll fluorescence is used as an indicator of cell viability, measure of photosynthetic capacity in marine phytoplankton as well as of plant response to stress factors. In living cells the intact chlorophyll molecules show red fluorescence, while the irreversible dammage of the cell is connected with a disappearance of fluorescence (Blates end Platt, 1984; van Hasselt, 1992; Ball et al., 1995; Balota, 1997). It should be noticed that the viability tests conducted by fluorescent microcopy are much more sensitive than the light microscopical ones.

The mechanism of the penetration of the fluorochromes RB and NR in the cell is based on the fact that walls of dead cells lose their semipermeability and these fluorochromes accumulate in cells. This mechanism of penetration is probably similar to the postmortal penetration of evans-blue, methylene blue and eritrosine (Gaff and Okong'O-Ogola, 1971) and are not based on cell viability as Levitt (1969) supposed. Moreover, these dyes are known as indicators which produce orange fluorescence and reveal the presence of vacuoles and lipid globules in the cells (Stanley and Linskens, 1974; Klut et al., 1988).

It is known that the fluorochrome CFW stains the cell walls (Nagata and Takabe, 1970; Waaland and Waaland, 1975; Kasten, 1981; Fisher et al., 1985; Klut et al., 1988) and produces blue fluorescence only in living cells (Herth and Schnepf, 1980).

It was established that CFR method infers cells viability by evaluating the integrity of the plasmalemma and testing for the presence of esterase, enzyme known to be essential for normal cell function. Active esterases that are associated with fully functional cell walls hydrolyze non-fluorescent FDA to yield fluorescein with bright yellow fluorescence in viable cells (Heslop-Harrison et al., 1984). Saga et al. (1987) reported statistically significant results and recommended FCR test as the most appropriate method for determining the percentage of living cells in suspensions from the unicellular alga *Entheromorpha intestinalis.*

In view of the need for a fast and precise evaluation of the physiological state of algal cultures, the aim of the present study was to evaluate the possibilities for testing the viability of different microalgae with the highly sensitive fluorescent microscopical methods. The most suitable viability tests were recommended depending on the morphological specificity of the various systematic groups studied.

Materials and Methods

Objects of the present study were old and fresh agar cultures of the following algal strains: *Anabaena variabilis, Synechocystis miniscula, Dunaliella salina, Scenedesmus acuminatus, Chlorella pyrenoidosa, Coelastrum cambricum, Chlamydomonas reinchardii, Asteromonas gracilis, Nephrochloris salina* and *Haematococcus gracilis.* The old cltures were stored for 9 months, while the fresh ones – 14 days at standard conditions.

The viability of algal cells was investigated after observing the fluorescence of chlorophyll and by aid of the fluorochromes: RB – 0.1 % solution in distilled water; NR – 0.01% solution in distilled water; CFW – 0.2% solution in distilled water; FDA – 2% solution in acetone. The dyes were added in quantity of 0.2 ml each one to the algal suspension so that their final concentration was 0.02, 0.002, 0.04 and 0.4%, respectively. The staining was carried out at room temperature for 30 min. Low concentration of fluorochromes and short incubation time were chosen to minimize the fluorochrome induced production of singlet oxigen in the light, which disrupt the homeostasis of the cytoplasm (Johnson and Martin, 1988; Russ et al., 1989).

Before observation, the stained sediments of algal suspensions were washed once with physiological solution and twice in the case of FDA.

The investigations were conducted by an Ergaval microscope with a fluorescent device. The red autofluorescence of chlorophyll was observed at 460–520 nm, the orange fluorescence following RB staining was observed at 560–580 nm, and the orange fluorescence after NR staining – at 460–520 nm. The blue fluorescence post staining with CFW was measured at 437–490 nm, and the yellow fluorescence after the use of the FCR – at 490–515 nm.

From each sample a total of 300 algal cells was estimated and the statistical significance of the results was computed by the Student's criterion.

Results and Discussion

Staining with rodamin B and neutral rot

The results obtained after staining with RB and NR were similar. In all objects studied, except *Anabaena variabilis,* in which no fluorescence was observed after staining with both fluorochromes, the dead algal cells showed orange fluorescence (Fig. 1). Some *Chlorella, Synechociytis, Coelastrum* and *Asteromonas* cells fluoresced homogenously with briliant lipid globules and vacuoles (Fig. 2).

The percentage of fluorescent cells recorded by aid of RB and NR was close to the percentage of cells which had lost the chlorophyll fluorescence, and the statistical differences were in most cases insignificant (Table 1).

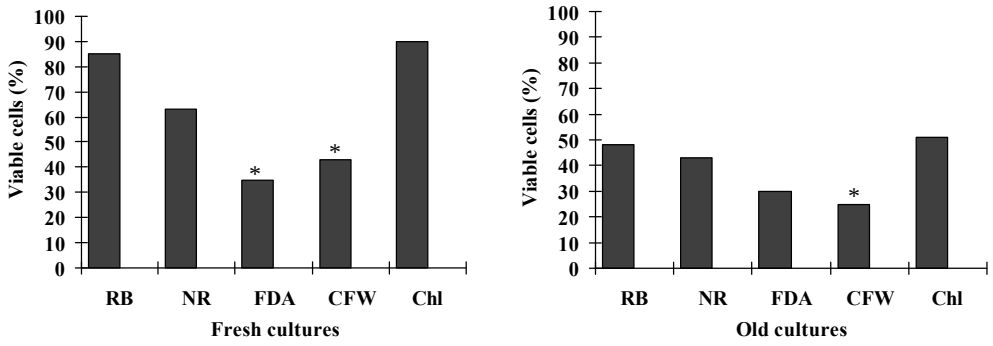

Fig. 1. Comparative investigation of the viability of old and fresh *Scenedesmus acutus* cultures by aid of the fluorochromes RB, NR, FDA, CFW and chlorophyll autofluorescence (* – statistically significant differences)

Table 1. Assessing the percentage of viability cells in algal cultures by aid of rodamin B and chlorophyll fluorescence

Algal cultures	Rodamin B (%)	Chlorophyll autofluorescence (%)
Dunaliella salina strain 212 (old)	68	63
Dunaliella salina strain 212 (fresh)	90	87
Dunaliella salina strain 209 (old)	29*	47*
Dunaliella salina strain 209 (fresh)	69	68
Chlorella pyrenoidosa (old)	23*	17*
Chlorella pyrenoidosa (fresh)	99	95
Scenedesmus acuminatus (old)	46	48
Scenedesmus acuminatus (fresh)	86	90

* statistically significant differences

Staining with calcofluor white

In fresh cultures CFW stained selectively the cell walls of living algal cells except *Anabaena variabilis* (Fig. 3). The intensity of fluorescence depended on wall thickness. The latter is age dependent and is a function of the ontogenetic phase of algal cells. Usually the brightest fluorescence was recorded in the mature cells. Differences in intensity of wall fluorescence were found in the genera *Chlorella, Coelastrum, Nephrochloris* and *Haematococcus.* Smaller differences were found in *Synechocystis* and *Asteromonas.* The age dependent differences after CFW staining could be recorded only with fluorimetry.

Following CFW treatment of *A. variabilis* cultures contrary to the other algal species under study, the cell walls of living cells were not stained, only the walls of dead empty cells showed a bright fluorescence. Besides, in old *Chlorella, Coelastrum*

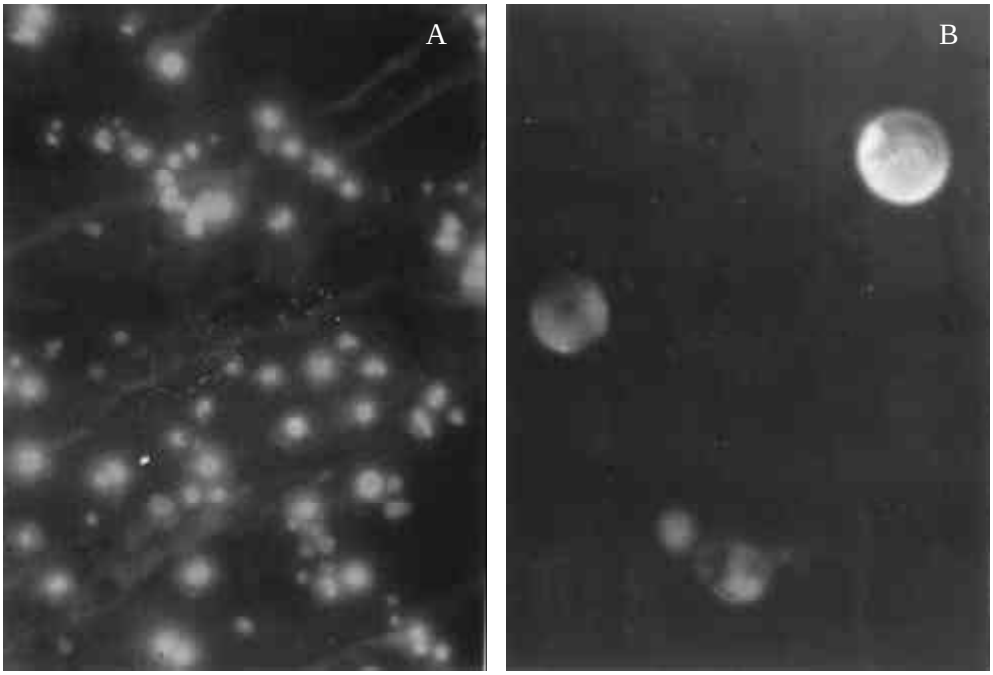

Fig. 2. Staining with rodamin B. A – *Chlorella pyrenoidosa*; B – *Nephrochloris salina*

and *Nephrochloris* cultures where all algal cells had not yet died, a fluorescence of the cell walls in 100% of the algal cells was also observed (Fig. 3A). At the same time the percentage of algal cells which have chlorophyll autofluorescence was between 65 and 75%. These results showed that the CFW-induced fluorescence could not be considered as an universal indicator for the viability of the algal cells.

Since the cell walls of developing *Scenedesmus, Chlorella, Asteromonas* and *Haematococcus* after CFW staining were strongly fluorescent (Fig. 3B), this staining could be recommended for investigation of autospore formation. It could be used as an indicator for the synchronization of algal cultures.

Staining with fluorescein diacetate

The majority of *Anabaena, Chlorella, Scenedesmus, Synechocystis, Coelastrum* and *Nephrochloris* cultures showed differentiation in staining of living and dead cells, which passed through a wide intermediary spectrum (Fig. 4). After performing the fluorochromatic tests, the bright chlorophyll fluorescence masked fluorescein fluorescence in the cytoplasm, because most of the algal species studied had large cup-like chloroplasts situated near the walls, over the entire cell surface. This fact prevents the proper recording of the viable cells in which the cytoplasm show fluorescence.

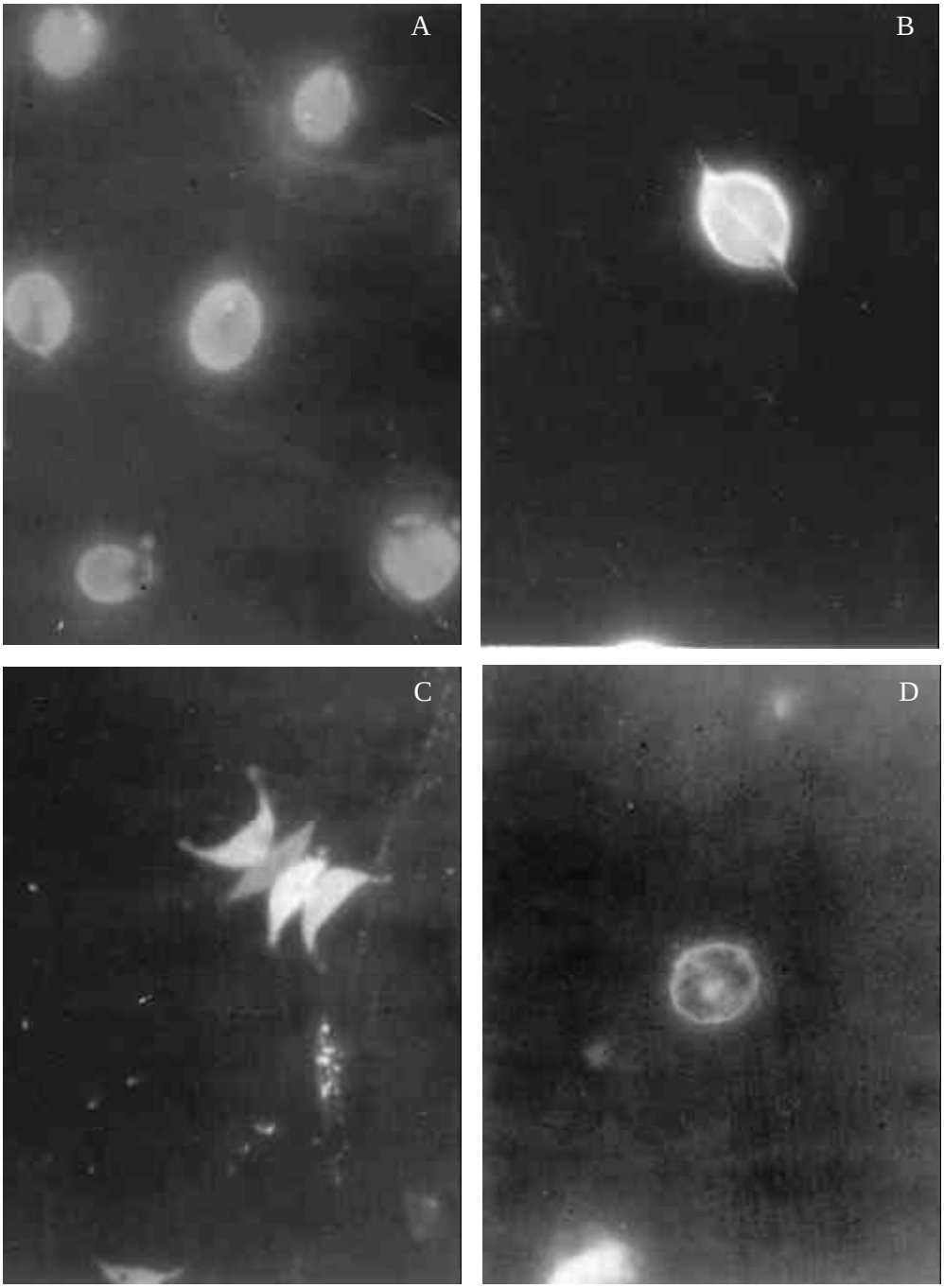

Fig. 3. Staining with calcofluor white. A – *Nephrochloris salina*; B – *Scenedesmus acuminatus* (mature cells with 2 autospores)*;* C – *Scenedesmus acuminatus* (cenobium)*;* D – *Haematococcus* (cell wall)

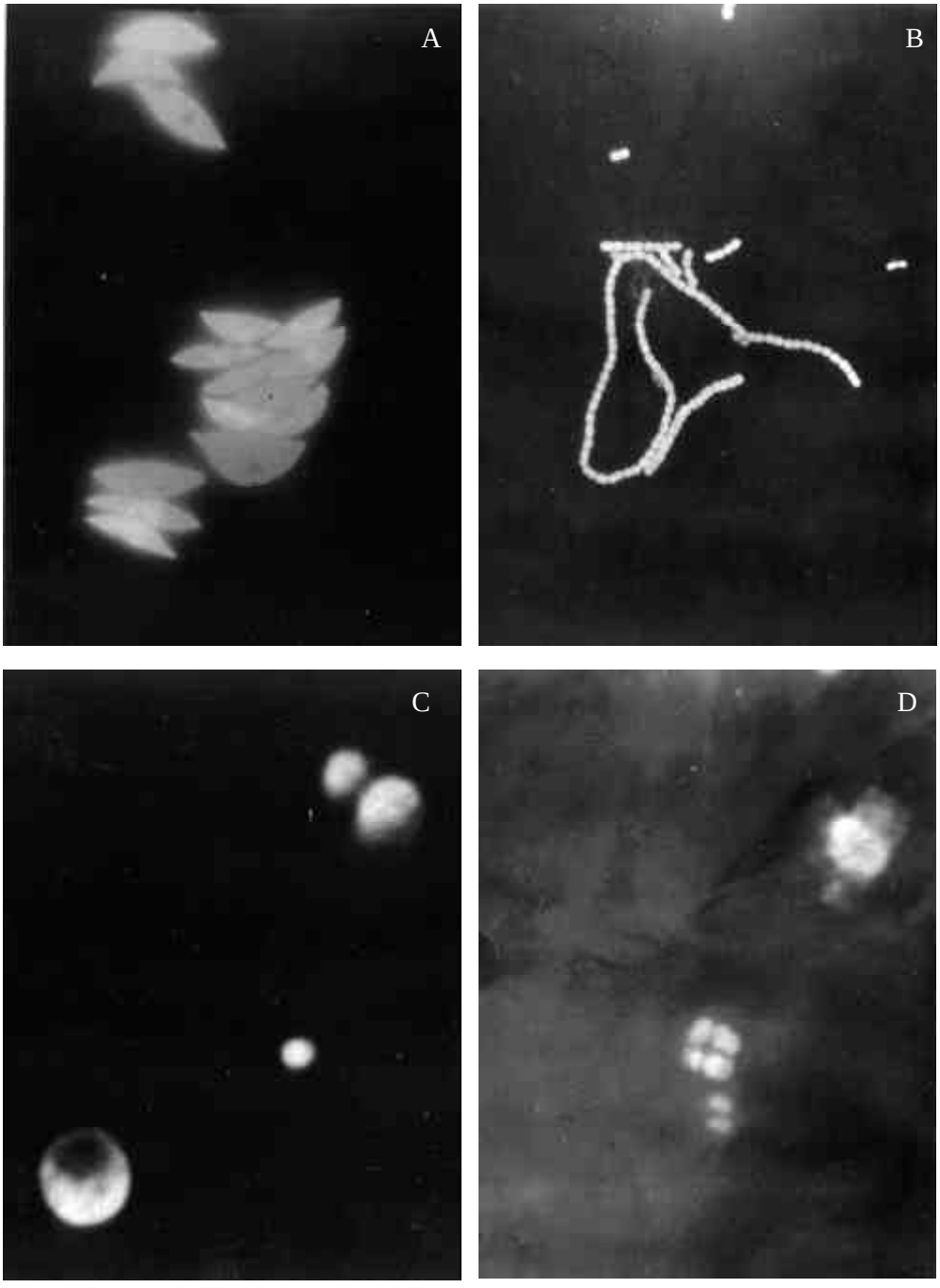

Fig. 4. Staining with fluorescein diacetate. A – *Scenedesmus acuminatus*; B – *Anabaena variabilis*; C – *Nephrochloris salina*; D – *Synechocystis minuscula*

In cells of bad physiological state where chlorophyll autofluorescence was lower, following FDA treatment in part of the cytoplasm a bright yellow fluorescence could be observed. Investigation of fresh *Anabaena* cultures (Fig. 4B) showed that FDA staining produced homogenous bright yellow fluorescence of the whole fibers. Chlorophyll fluorescence was weak, due to its low content in the representatives of division *Cyanophyta* and it did not mask the fluorescence caused by FDA. Therefore, application of FCR test could be recommended for assessment of viability in algae from division *Cyanophyta.* According to data reported by Saga et al. (1987), FDA was also suitable for the viability determination of the green marine alga *Entheromorpha intestinalis,* which had small lens-like chloroplasts.

Assessment the physiological state of algal cultures with fluorochromes

Comparison of the results concerning algal viability evaluated by different dyes revealed that RB and NR gave values close to the values registered for the chlorophyll autofluorescence (Fig. 1).

Results of the present study confirmed that staining with the fluorochromes under study helped to distinguish not only the living and dead cells, but a number of intermediary states as well. The ability of the FDA to assess the damaged cells or cells with affected metabolic activity which are characterized by yellow fluorescence of only a part of the cytoplasm is of particular importance. The observed algal cells with a low viability are in concert with the opinion of Jones (1987a,b), who proved that cells are desactivated before the true cell death occur.

The investigations of other authors had been devoted to the use of fluorochromes for the cytochemical characterization of phytoplankton and to the identification of various cell components. Morphological changes of some cell components in various metabolic states had been recorded (Klut et al., 1988; Klut et al., 1989). These authors underlined that fluorochrome effect after staining of plankton algae, belonging to 4 different taxonomic divisions, depend not only on experimental conditions and type of cells, but on chemical and physiological differences.

The present investigation showed that a wide spectrum of possibilites exists for the evaluation of algal viability by use of fluorochromes.

After assessment of the applicability of different fluorescent microscopical tests to estimate the viability of wide range of microalgal taxons, it was established that chlorophyll autofluorescence could be accepted as the most convenient method for differentiation between living and dead algal cells. Since in most of the algal species investigated the chlorophyll autofluorescence masked cytoplasmic fluorescence, the tests based on fluorescence of the living cytoplasm (as in the case of the FCR test and CFA) proved to be inappropriate. The tests based on the fluorescence of the dead cell cytoplasm (NR and RB) appeared to be useful for vitality assessment, because chlorophyll fluorescence was lost and did not mask cytoplasmic fluorescence.

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