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F. J. Jochem

Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate

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Abstract Cytometric quantification of cellular fluorescence upon cleavage of fluorescein diacetate (FDA) is presented as a sensitive and rapid technique to assess phytoplankton metabolic activity during exposure to prolonged darkness of 10 to 12 d. Two distinct types of metabolic response to darkness are distinguished: Type I cells (*Brachiomonas submarina*, *Pavlova lutheri*, *Chrysochromulina hirta*) adapt to prolonged darkness by reducing their metabolism to a lower level of activity (~10% of initial in *P. lutheri*, *C. hirta*, ~0.5% in *B. submarina*) within few days, whereas Type II cells (*Prymnesium parvum*, *Bacteriastrum* sp., unidentified pennate diatom) continue with unchanged activity. Type I cells were able to maintain their initial cell abundance and commenced rapid cell growth upon re-illumination after 12 d of darkness. Among Type II cells, diatoms were able to maintain cell abundance and growth capacity as well, whereas *P. parvum* was not. Type I cells are expected to exhibit competitive advantages in environments with frequent or long dark periods. Bacterivory further supports dark survival in *C. hirta*.

Introduction

Although photoautotrophic growth of phytoplankton is light dependent and therefore restricted to the euphotic zone, live algal cells are often encountered well below the lit ocean layers. These cells might have sunken out of the upper water column or occurred in physically displaced surface-near water. Observations of deep phytoplankton assemblages have been presented as evidence for water-mass subduction in the California Current System (Kadko et al. 1991; Washburn et al. 1991), for example. Murphy and Cowles (1997) reported 50 × 60 km wide patches of live phytoplankton at aphotic depths of 150 to 200 m off the California shelf that contributed a biomass about 2.5 times the amount of chlorophyll in the overlaying euphotic zone. Especially during winter and spring, phytoplankton of temperate and boreal regions can experience quite frequent deep-mixing, due to strong winds and thermal convection.

The time of withstanding deep-mixing and dark conditions can determine the survival and competitive success of a specific population or species in an environment exhibiting such conditions frequently. Some groups of phytoplankton possess the capability of forming resting cells such as resting spores in diatoms, cysts in dinoflagellates and akinetes in cyanobacteria. The majority of small eukaryotic phytoflagellates, however, do not show such survival stages. Because of their small size of mostly <10 μm, they cannot be expected to contain enough energy reserves to survive long periods of darkness without either switching to a heterotrophic mode of nutrition or reducing their metabolic activity to a necessary minimum. The study of physiological response in these species is vital to understand the ecological role of deep-water chlorophyll accumulations.

The measurement of fluorescein diacetate (FDA) hydrolysis has been applied to estimate microbial biomass on coniferous needles (Swisher and Carroll 1980), to determine total microbial activity in soil (Schnürer and Rosswall 1982), water (Holzapfel-Pschorn et al. 1987) and deep-sea sediments (Köster et al. 1991; Gumprecht et al. 1995), and to differentiate between live and dead/unhealthy cells in mammalian cell cultures (Ross et al. 1989) and microalgae (Gilbert et al. 1992). In connection with pollution studies and using fluorescence microscopy, Bentley-Mowat (1982) first reported

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F.J. Jochem
Institut für Meereskunde,
Düsternbrooker Weg 20,
D-24105 Kiel, Germany

Corresponding address:
The University of Texas at Austin,
Marine Science Institute,
750 Channel View Drive,
Port Aransas, Texas 78373, USA
e-mail: frank@emcs.de

that the intensity of fluorescence derived from the cleavage of FDA appeared to depend on the "metabolic vigour" of the cells. Dorsey et al. (1989) optimized the FDA technique for application in flow cytometry; their protocol was adopted here except for minor changes described below.

FDA is a nonpolar, nonfluorescent substance which enters the cells freely. Inside the cell, nonspecific esterases, among them lipase and acylase but not acetylcholinesterase (Guilbaut and Kramer 1966), break the FDA molecule into one brightly fluorescing fluorescein and two acetates. Being highly polar, the fluorescein is trapped within cells exhibiting cell membrane integrity, and the amount of fluorescence will therefore increase over time depending on the metabolic activity of those esterases.

Materials and methods

The chlorophyte *Brachiomonas submarina*, the prymnesiophytes *Prymnesium parvum, Pavlova lutheri*, and *Chrysochromulina hirta* (Baltic Sea isolate), the diatom *Bacteriastrum* sp. and an unidentified pennate, *Nitzischa*-like diatom isolated from the North Atlantic (47°N; 20°W, R.V. "Meteor", May 1992) were maintained as batch cultures in *f*/20 medium in artificial seawater (except for *C. hirta* in Baltic Sea water) at 18 °C and 80 μE m^{-2} s^{-1} provided by white fluorescent tubes under a 14 h light:10 h dark photocycle. For the dark survival experiments, subsamples of late log-phase cultures were inoculated into fresh *f*/20 medium, placed into darkness at 10 °C, and sampled daily at noon of the previous photocycle.

To test for heterotrophic potentials during dark survival, in Experiment I, comprising *Brachiomonas submarina*, *Pavlova lutheri*, *Chrysochromulina hirta*, *Prymnesium parvum* and the pennate diatom, cultured bacteria were added to a final concentration of 7.5×10^5 ml^{-1} in parallel incubations to those without bacteria, except for the diatom where bacterivory was not expected. In Experiment II, comprising *B. submarina*, *P. lutheri* and *Bacteriastrum* sp., parallel incubations with added organics (final conc. 5 μ*M* of glucose and leucin) should test for osmotrophic potentials.

A stock solution of FDA (Sigma Chemicals F-7378) of 5 mg ml^{-1} was made in dimethylsulfoxide (DMSO) and stored at 4 °C. The stock solution was thawed (DMSO freezes at 18 °C) and diluted 100-fold in distilled water; since FDA is only slightly soluble in aqueous solutions and tends to flocculate at >1 μg ml^{-1}, the stock solution was injected rapidly into the ice-cold water and mixed quickly. Although the working solution might appear slightly opaque, flocculation was prevented. The working solution was kept on ice to minimize FDA degradation, for a maximum of 3 h, and prepared fresh daily. A total of 100 μl FDA working solution was added to 3 ml of sample in Becton-Dickinson (BD) cytometer tubes kept at room temperature in the dark until measurement.

Cells were analyzed by a BD FACSort flow cytometer equipped with a 488 nm argon laser. Phytoplankton cells were identified and distinguished from other particles by gating on two-parameter-plots of Forward Angle Light Scatter (FSC) versus chlorophyll fluorescence gathered through a 650 nm longpass filter. Green fluorescein fluorescence was measured through a 535 ± 15 nm bandpass filter on a four-decades log scale. BD Calibrite standard beads with green and red fluorescence, respectively, were used to calibrate the machine settings for identical fluorescence yields of the daily analyses, and all FDA fluorescence measurements were taken for all species and all days at the same settings, so that FDA fluorescence readings normalized per cell are comparable among the studied species.

Results

For optimization and standardization of the FDA staining protocol, accumulation of fluorescein was followed continuously for 15 min after FDA addition in pre-experiments (Fig. 1). FDA readily penetrated into the cells right after addition, and green fluorescence increased for 4 to 5 min. Thereafter, variation in cellular fluorescence increased, and eventually leakage of fluorescein out of the cells further increased the variation and reduced the mean cellular fluorescence. For the subsequent dark survival studies it was decided to take fluorescence readings exactly 5 min after FDA addition. This timing gave a good compromise of reasonably low variation in fluorescence readings and high fluorescence yields from FDA cleavage. Instrument settings were adjusted so that non-marked cells exhibited a "fluorescence" below 2 relative units on the four-log scale. Hence, cells with fluorescence readings above this value were considered FDA positive.

Generally, all cultures exhibited a fairly low variation in their FDA fluorescence at the start of dark incubations (Fig. 2a, b; first measurements). As the experiments progressed, FDA fluorescence not only decreased but in some cases the coefficient of variation of the fluorescence frequency distributions increased as well as can be seen by broader frequency distributions in Fig. 2a. For the presentation of cellular metabolic activity as assessed by FDA fluorescence, both mean fluorescence per cell and the percent of FDA positive cells were considered. Despite somewhat broader frequency distributions in FDA fluorescence among some of the tested cultures, mean and median values generally displayed the same results.

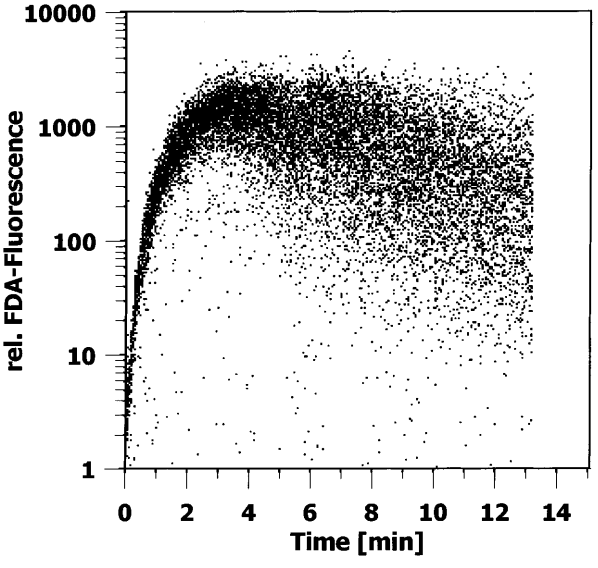

Fig. 1 Time course of fluorescence (relative units) accumulation upon FDA addition in *Brachiomonas submarina* as revealed by flow cytometry

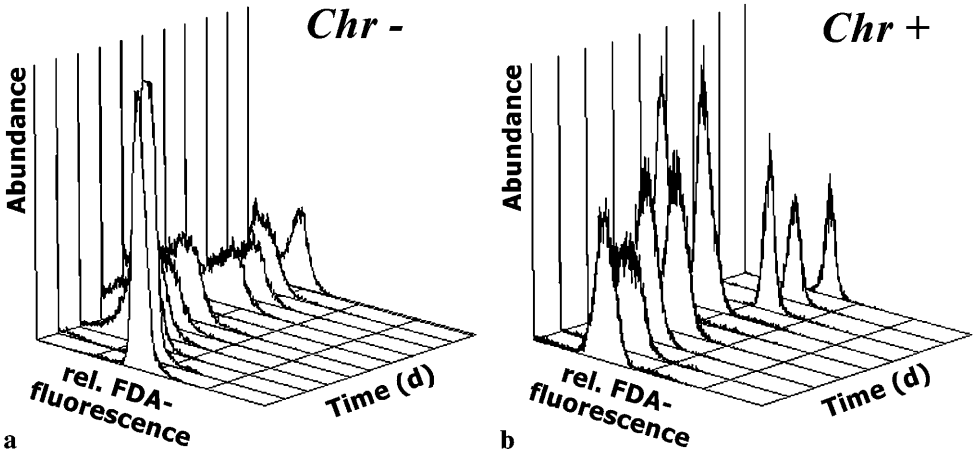

Fig. 2 Daily frequency distributions of FDA-derived fluorescence (relative units) in *Chrysochromulina hirta* kept in darkness **a** without (*Chr*−) and **b** with (*Chr*+) addition of bacteria

Following the mean cellular fluorescence during dark cultivation reveals two different principles of metabolic response. *Brachiomonas submarina*, *Pavlova lutheri* and *Chrysochromulina hirta* represent the first type (Fig. 3a): after some reduction in FDA fluorescence during the first days there was a distinct, almost one magnitude downward step after 4 d (*P. lutheri*) to 6 d (*B. submarina*) in the dark. Subsequently, metabolic activity remained on a more-or-less constant low level. *C. hirta* reduced its metabolic activity already during the first 4 d to reach a constant low level thereafter. In contrast, *Prymnesium parvum* and *Bacteriastrum* sp. kept their metabolic activity at an unchanged magnitude throughout the experiment (Fig. 3b).

With the addition of bacteria, metabolic activity of *Chrysochromulina hirta* and *Pavlova lutheri* initially decreased as described above but increased again after Day 4 (Fig. 3c). The comparison of distinct FDA fluorescence frequency distributions in *C. hirta* (Fig. 2) reveal that without bacteria addition, the mean fluorescence decreased and variation of fluorescence increased, meaning that metabolic activity decreased faster in some cells than in others (Fig. 2a). In the presence of bacteria, the culture kept a narrow frequency distribution throughout the experiment, suggesting that all cells responded equally to the presence of bacteria (Fig. 2b). In the other species, the addition of bacteria altered neither the type of response nor the magnitude of low-level esterase activity (Fig. 3c).

The cultures without added bacteria were re-illuminated after 13 d in the dark. In Type I response species, metabolic activity increased upon re-illumination, a response which was most pronounced in *Brachiomonas submarina* (Fig. 3a). No change in metabolic activity upon re-illumination was recorded in *Bacteriastrum* sp., whereas *Prymnesium parvum* exhibited a decrease in FDA fluorescence.

The response in the fraction of FDA positive cells was more heterogeneous (Fig. 4a, b): *Pavlova lutheri* and *Chrysochromulina hirta* exhibited an initial decrease but a later recovery which reached >90% in the latter and near 80% in *P. lutheri*. Both *Brachiomonas submarina* and *Prymnesium parvum* experienced a steady decline in the contribution of metabolically active cells throughout darkness which developed faster in the former species; re-illumination caused a fast increase of the FDA+ fraction to >90% within 2 d in both species. *Bacteriastrum* sp. kept its FDA+ fraction at nearly 100% throughout the experiment. With the addition of bacteria, the FDA+ fraction remained at 90 to 100%, except in *P. parvum* which showed the same decrease as mentioned above (Fig. 4c).

Cell numbers in Type I response species (Fig. 5a) remained quite constant during the first 10 d of darkness. Subsequently, *Pavlova lutheri* showed a slight and *Chrysochromulina hirta* a strong decrease in abundance, whereas *Brachiomonas submarina's* abundance still remained constant. Population growth commenced immediately upon re-illumination in *C. hirta*, but seems to have lagged until Day 17, the end of the experiment, in both *B. submarina* and *P. lutheri*. *Bacteriastrum* sp. abundance remained unchanged during darkness and took off right after re-illumination, whereas *P. parvum* cell numbers declined steadily and did not recover after re-illumination (Fig. 5b). Addition of bacteria did not alter the pattern of cell abundance (Fig. 5c).

The second dark survival experiment generally revealed the same response patterns and time frames (Fig. 6a to c). Metabolic activity decreased after Day 3 in *Pavlova lutheri* and Day 7 in *Brachiomonas submarina* whereas that of *Bacteriastrum* sp. remained unchanged. The fraction of FDA+ cells dropped after 3 d in *P. lutheri* and 7 d in *B. submarina* but remained near 100% in *Bacteriastrum* sp. In this experiment, cell numbers of *B. submarina* decreased slightly, *Bacteriastrum* sp. abundance remained unchanged, and *P. lutheri* exhibited slight growth during the first 4 d in darkness. The addition of glucose and leucin had no influence on any of the species' responses.

Discussion

The FDA method appears as a sensitive, simple and rapid technique to assess cell-to-cell as well as temporal

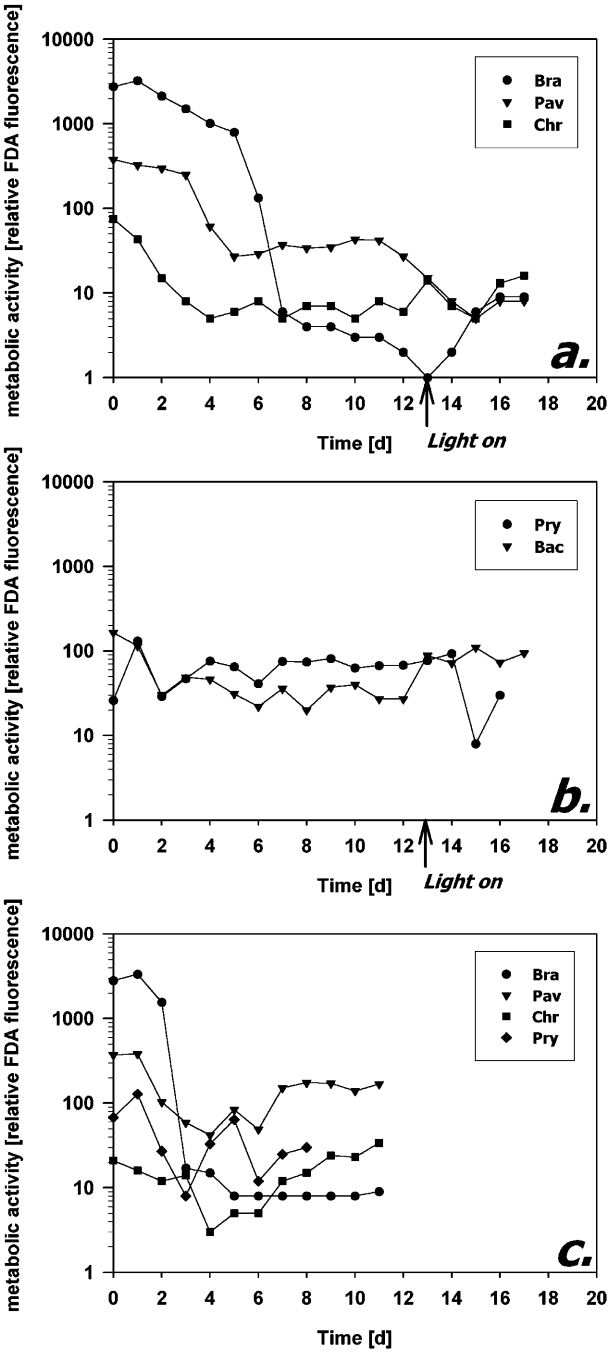

Fig. 3 Mean cellular FDA-derived fluorescence (relative units) of phytoplankton kept in darkness; **a, b** incubation without bacteria, **c** incubation with added bacteria (Bra, *Brachiomonas submarina*; Pav, *Pavlova lutheri*; Chr, *Chrysochromulina hirta*; Pry, *Prymnesium parvum*; Bac, *Bacteriastrum* sp.; *arrows* in a and b indicate time of re-illumination in incubations without bacteria)

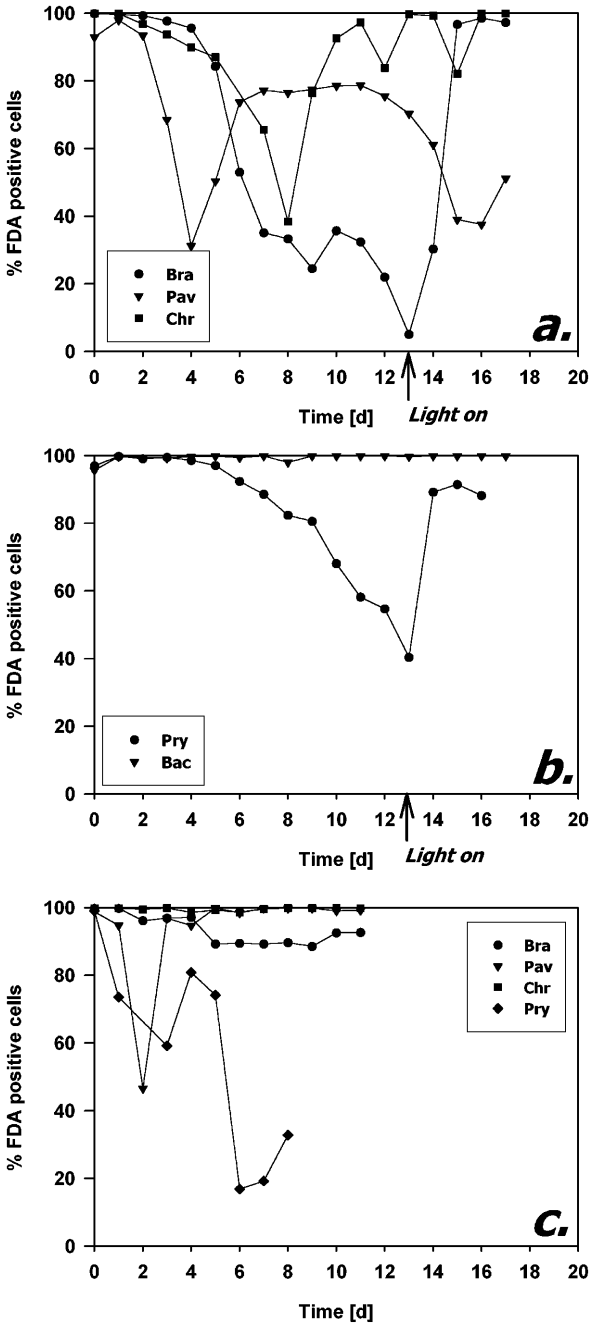

Fig. 4 Fraction of FDA-positive cells (%) of phytoplankton kept in darkness; **a, b** incubation without bacteria, **c** incubation with added bacteria; species names as listed in Fig. 3 (*arrows* in a and b indicate time of re-illumination in incubations without bacteria)

variability in metabolic activity of microalgae. The presented protocol worked well with all tested species of different algal classes and gave reproducible results. Successful application of same or similar protocols has been reported for a total of 44 different phytoplankton species from all major unicellular algal groups (Selvin et al. 1988; Dorsey et al. 1989; Gilbert et al. 1992; Geary et al. 1998; present study) as well as successful bulk measurements of marine field samples to test for the toxicology of weed-killers, insecticides and metals (Gilbert et al. 1992).

Since the esterases enabling the FDA assay turn over within a time frame of several hours (Yentsch et al. 1988), this technique seems appropriate to detect changes in metabolic activity on a day-to-day time scale. This is supported by results of Geary et al. (1998), who were

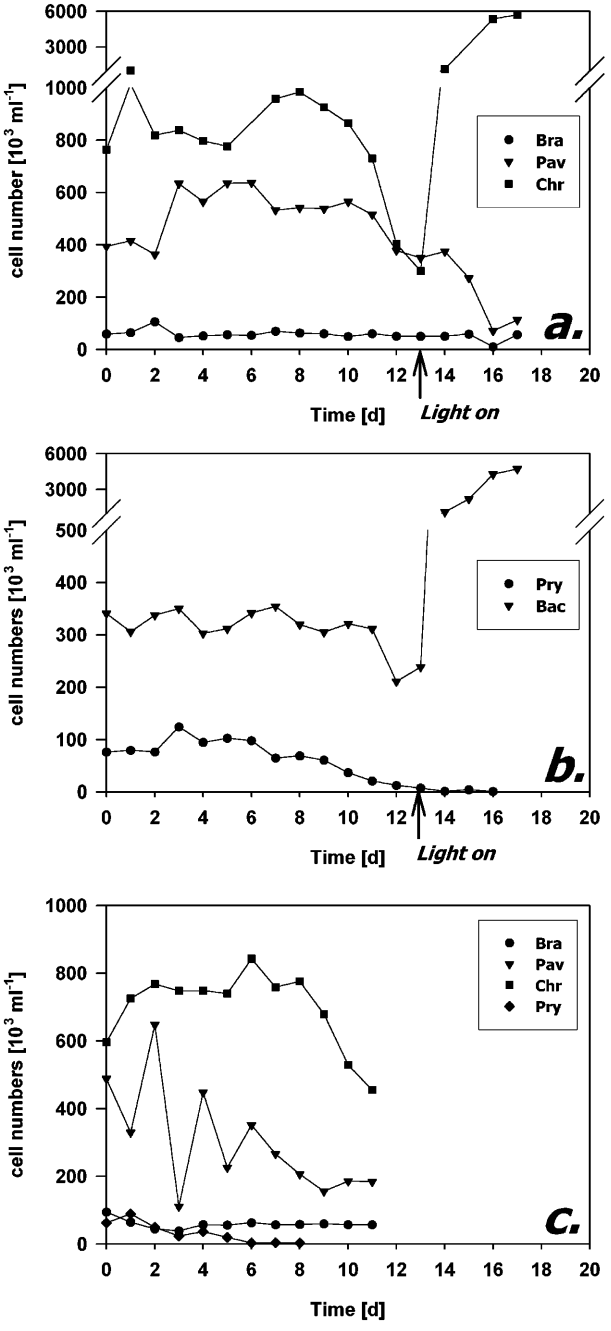

Fig. 5 Cell abundance (10^3 ml^{-1}) of phytoplankton kept in darkness; **a, b** incubation without bacteria, **c** incubation with added bacteria; species names as listed in Fig. 3 (*arrows* in a and b indicate time of re-illumination in incubations without bacteria)

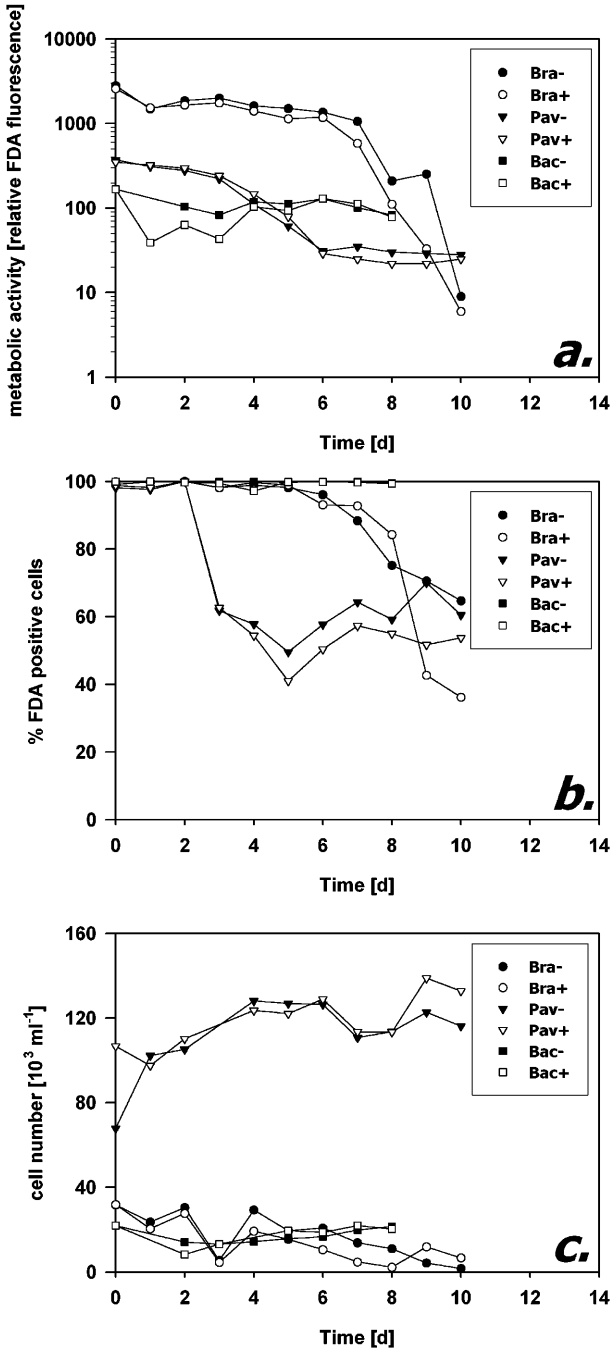

Fig. 6 **a** Mean cellular FDA-derived fluorescence (relative units), **b** fraction of FDA-positive cells (%), and **c** cell abundance (10^3 ml^{-1}) of phytoplankton kept in darkness [Bra, *Brachiomonas submarina*; Pav, *Pavlova lutheri*; Bac, *Bacteriastrum* sp.; *filled symbols* represent incubations without; *open symbols* those with addition of glucose and leucin (final conc. 5 μM)]

able to distinguish cyanobacteria (*Microcystis aeruginosa*) grown under different light intensities and under phosphate replete/deplete conditions after 2 d of adaptation; the light-covarying FDA fluorescence corresponded to respectively different cell growth rates. Dorsey et al. (1989) report covariation of FDA assays and $^{14}CO_2$-fixation comparing eight algal species. The FDA assay is, thus, not only helpful to discriminate "healthy" and "stressed" cells but also to quantify subtle responses to environmental impacts.

The advantage of cytometric measurements over fluorescence microscopy or bulk estimates by fluorometry lies in the assessment of minor changes in metabolic activity by the detection of changes in the amount of fluorescence accumulated per time unit on a single cell basis. This ability might even allow the differentiation of metabolic responses to environmental factors by differ-

ent species/populations in one given sample. Bulk measurements (e.g. fluorometry, automated measurement in microtitration plates) must account for fluorescence originating from spontaneous degradation and/or bacterial cleavage of FDA as well. These effects were not important to cytometric analyses as only fluorescein fluorescence related to individual algal cells was recorded. This specific advantage of flow cytometry suggests that changes in FDA cleavage upon addition of bacteria during dark survival reflect the phagotrophic potential of the studied algae.

The analysis of metabolic activity revealed two distinct types of physiological response to prolonged darkness among the studied phytoflagellates. The species assigned to Type I (*Brachiomonas submarina*, *Pavlova lutheri*, *Chrysochromulina hirta*) seem to recognize the problem of darkness and energy limitation and react by reducing their metabolic activity within a few days. Thereby, these species were able to sustain their population abundance for 2 weeks in the dark. It should be noted that the result of lower cellular FDA fluorescence only applies to cells acknowledged as FDA positive. Therefore, the Type I response represents the metabolic response in active cells only and is not influenced by the fact that the fraction of active (FDA +) cells decreased concomitantly.

The second type of response (*Prymnesium parvum*, *Bacteriastrum* sp., pennate diatom) lacks an adjustment in metabolic activity upon darkness. In the case of *P. parvum*, going on "as usual" results in constant FDA readings but an inevitable decrease in cell abundance. Although the fraction of FDA-positive cells increases drastically upon re-illumination after 2 weeks of darkness, it seems that the surviving cells need the new energy to refill their exhausted cellular reserves before they can eventually divide. Therefore, in contrast to Type I species, re-illumination did not result in population growth.

The two diatoms exhibited a peculiar pattern in that their metabolic activity did not change and almost all cells remained metabolically active. Still they were able to sustain their cell abundance in the dark and to commence rapid population growth upon re-illumination. Diatoms, particularly polar species that are exposed to long winter darkness, are known for their dark survival potentials (Antia and Cheng 1970; Palmisano and Sullivan 1982). Their strategies comprise reduction of metabolism (French and Hargraves 1980), formation of resting spores (Durbin 1978; Doucette and Fryxell 1983) or resting cells without morphological differences to vegetative cells (Anderson 1975; Hargraves and French 1983), and facultative heterotrophy (Hellebust and Lewin 1977).

Neither of the studied species displayed signs of resting spore formation, which besides morphological changes would have resulted in severely reduced FDA fluorescence as well. Eight diatoms from the Southern Ocean (Peters and Thomas 1996) and three diatoms from the North Sea (Peters 1996) survived up to 10 months of darkness without spore formation and kept their potential for high photosynthesis during dark incubation. *Thalassiosira weissfloggii* survived 2 months of darkness without spore formation and commenced exponential growth upon re-illumination (Murphy and Cowles 1997); the authors suggest that both the photochemical apparatus and biochemical carbon fixation pathways remained functional and >80% of cells remained metabolically active as microscopically derived from FDA assays. From measurements of particulate organic carbon they further assume that *T. weissfloggii* utilized organic carbon during dark survival.

In the present experiment, the inability to take up added organics does not suggest heterotrophy. This observation does not, however, exclude a potential for heterotrophy. The conducted dark experiment might have been too short to yield a physiological state of deprivation dire enough to initiate heterotrophy, and energy reserves might have been sufficient to sustain unchanged metabolic activity without biasing the ability to commence rapid cell division upon re-illumination. The nature of the physiological mechanism providing remarkable dark survival capabilities and sustaining unreduced metabolic activity at the same time still remains to be resolved.

From their results, the prasinophyte strain CCMP Ω48-23 can also be assigned to Type I (Dorsey et al. 1989). Among three dinoflagellates from the Ria de Vigo, Spain, tested for dark survival *Protogonyaulax affinis* was unable to survive for even a few days, *Gymnodinium catenatum* took advantage of cyst formation, but *Prorocentrum lima* was still alive after 3 weeks (Selvin et al. 1988); its lower FDA fluorescence in the dark than in the light as concluded from qualitative microscopic analyses makes this species likely to be of Type I as well and the most successful dinoflagellate in their dark survival assay.

It can be assumed that Type I species will gain a competitive advantage over Type II species in environments where long and/or frequent dark conditions might be encountered. Reduction of metabolic activity to preserve scarce energy resources is essential for survival under such circumstances. Type II algae would eventually run out of energy and grow themselves to death. Since high metabolic activity can be regained pretty fast, at least in some Type I species (see Fig. 3a, notably *Brachiomonas submarina*; also Ω48-23 in Dorsey et al. 1989), a Type II strategy does not seem advantageous in environments of short dark periods either (such as frequent shallow mixing that quickly returns cells to the surface).

In addition to energy saving, energy scavenging could be a complementary survival strategy. Previous studies showed that phytoplankton at the lower boundary of the euphotic zone might supplement a part of their carbon requirement by osmotrophy (Vincent and Goldman 1980) or phagotrophy (Bird and Kalff 1989). The more "phytoflagellates" studied, the more that turned out to be facultative or obligate heterotrophs. Often, heterotrophic modes of nutrition in phytoplankton are stimulated by

darkness, as for example shown for the dinoflagellate *Heterocapsa triquetra* (Legrand et al. 1998).

Among the species studied here, *Chrysochromulina hirta* exhibited clear signs of enhanced dark survival and higher metabolic activity when bacteria were added to the dark culture, pointing towards bacterivory in this species (see Fig. 2b). Several species of *Chrysochromulina*, for example *C. brevifilum* (Jones et al. 1995) and *C. polylepis* (Nygaard and Tobiesen 1993; Rhodes et al. 1994), exhibited bacterivory under light and/or nutrient limitation, whereas phagotrophy was absent in *C. quadrikonta* and *C. camella* (Rhodes et al. 1994). Phagotrophy in *C. hirta* was previously documented by Kawachi et al. (1991). Providing that the FDA results in fact reveal bacterivory in *C. hirta*, this species thus exhibits a double competitive advantage for dark survival: Type I metabolic activity response and the ability to utilize resources do not dependent upon light.

Whereas phagotrophy was demonstrated in *Prymnesium patelliferum* (Tillmann 1998) and *P. parvum* (Nygaard and Tobiesen 1993), the culture of *P. parvum* studied here showed no sign of darkness-induced phagotrophy. *Brachiomonas submarina* did not seem to utilize bacteria or organics either, though such growth capabilities have been reported (Tsavalos and Day 1994).

Whereas live phytoplankton accumulations well below the euphotic zone seem to occur quite frequently in different parts of the world's oceans, we still lack a profound knowledge of their origin, species composition and the physiological adaptations of the involved algae. The extended deep-water patch of chlorophyll off the California shelf (Murphy and Cowles 1997) was not taxonomically analyzed, but the authors assume that it originated from a sedimented diatom-bloom. High chlorophyll accumulation at 150 m depth, well below the euphotic zone in the Polar Front of the Southern Ocean in austral spring 1992, could be attributed to diatoms as well, namely *Corethron cryophilum* (Jochem and Meyerdierks unpublished). Algal communities living and proliferating at aphotic and anoxic depths in the central Baltic Sea, however, were composed of various <10 μm phytoflagellates, some resembling *Chrysochromulina* sp. (Detmer et al. 1993). The cytometric FDA protocol might provide a useful tool to further investigate the physiology of algal cells in aphotic depths and may reveal the ubiquity of the two established types of metabolic adaptation to dark survival. This task is further eased by the recent development of a protocol for cryopreservation of FDA-labeled phytoplankton cells (Faber et al. 1997).

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