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Delayed luminescence of *Prorocentrum minimum* under controlled conditions

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Abstract

Delayed luminescence (DL), also termed delayed fluorescence or delayed light emission, is the phenomenon of long-lived light emission by plants and cyanobacteria after being illuminated with light and put into darkness. Culture growth of three *Prorocentrum minimum* strains was studied with DL measurements. DL decay kinetics was measured from 1–60 s after a pulse of white light. The strains used were from the Adriatic Sea (PmK), from Chesapeake Bay, USA (D5), and from the Baltic Sea (BAL), cultured at salinity of 32, 16, and 8 (practical salinity scale), respectively. The strains differed in cell size and chlorophyll *a* content (PmK > D5 > BAL), as well as in DL parameters. The DL results were compared to standard measurements of culture density and carbon content (calculated from biovolumes). DL decay curves had a specific peak, which changed with culture growth and showed more similarities between the strains PmK and D5. The DL intensity increased with cell density and carbon content in a two-stage process, corresponding to the lag and exponential phases of growth. DL intensity was best correlated with carbon content irrespective of strain and is proposed as an estimate of biomass and for differentiating between lag and exponential phases of growth.

Keywords: *Prorocentrum minimum*; Delayed luminescence; Growth phases

1. Introduction

Prorocentrum minimum (Pavillard) Schiller is a common bloom-forming dinoflagellate in temperate waters and is potentially toxic (Okaichi and Imatomi, 1979; Shimizu, 1987; Grzebyk et al., 1997; Denardou-Queneherve et al., 1999). It has a wide geographical distribution and its blooms generally occur in zones affected by freshwater and/or anthropogenic inputs (Grzebyk and Berland, 1996; Witek and Plinski, 2000). The ability of *P. minimum* to survive and grow under nutrient- and light-stressed conditions has been

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studied in several field (Tyler and Seliger, 1981; Harding, 1988; Harding and Coats, 1988; Stoecker et al., 1997) and laboratory investigations (Faust et al., 1982; Coats and Harding, 1988; Sciandra, 1991; Grzebyk and Berland, 1996).

Growth of *P. minimum* shows no strict requirements for temperature, light, or salinity conditions (Grzebyk and Berland, 1996). It grows in a temperature range between 8 and 31 °C, with an optimum between 18 and 26.5 °C. Variations in light intensity between 30 and 500 μmol photons m^{-2} s^{-1} has little effect on its growth. The optimal salinity range is from 15 to 35 PSU, and, after adaptation, slow growth occurs at salinities as low as 5. Higher growth rates can be observed at intermediate salinities (15–17 PSU; Hajdu et al., 2000).

Delayed luminescence (DL), also termed delayed fluorescence or delayed light emission, is the phenomenon of long-lived light emission by plants and cyanobacteria after being illuminated with light and placed in darkness (Strehler and Arnold, 1951). In contrast to rapid fluorescence, which is light emission on the nanoseconds time-scale, the characteristic times for DL are from milliseconds to seconds. The emission spectrum resembles the fluorescence emission spectrum of chlorophyll *a* (Arnold and Davidson, 1954; Van Wijk et al., 1999). DL originates from the repopulation of excited states of chlorophyll from stored energy after charge separation (Jursinic, 1986), whereas rapid fluorescence reflects the radiative de-excitation of excited chlorophyll molecules before charge separation.

The main source of DL is photosystem II (PSII) in the thylakoid membrane of chloroplasts. Charge pairs are generated in PSII during illumination, with positive charges located on the oxygen-evolving complex (OEC) and negative charges on quinone acceptors (Q_A and Q_B). The slow components of DL originate in back reactions between the S_2 and S_3 states of the OEC and quinones Q_A and Q_B (Joliot et al., 1971). The half-times of these reactions in isolated chloroplasts are 1.5 s for $Q_A + S_{2/3}$ and 25 s for $Q_B + S_{2/3}$ (Rutherford and Inoue, 1984). DL has hyperbolic decay kinetics in the first seconds (Scordino et al., 1996), sometimes followed by a more or less pronounced peak (Milani et al., 2003). Desai et al. (1983) observed the peak after illuminating the sample with far red light. One or more transient peaks were induced also by phosphorus starvation and carbon dioxide deficiency (Mellvig and Tillberg, 1986).

Delayed luminescence intensity (DLI) represents the integral under the decay curve and is an increasing function of the number of PSII centers, the fluorescence yield, and the rate of back reactions, which are influenced by the membrane potential and pH gradient (Joliot et al., 1971; Wraight and Crofts, 1971; Avron and Schreiber, 1979; Joliot and Joliot, 1980). In field studies, DLI was reported to be a measure of photosynthetic activity (Schneckenburger and Schmidt, 1996). Krause and Gerhardt (1984) and Wiltshire et al. (1998) have shown that DL can be applied in limnology and oceanography and that DLI of phytoplankton is a measure of living algal biomass. Yacobi et al. (1998) have analyzed natural phytoplankton samples using DL excitation spectra and were able to detect taxonomical changes in the algal communities. This technique is now used in monitoring freshwater phytoplankton (Hakanson et al., 2003). To our best knowledge, no studies have yet been published on *P. minimum* DL kinetics and DLI variation during different growth phases.

The aim of the present study was to monitor the changes in DLI of *P. minimum* during culture growth and to qualitatively compare DL decay curves of three *P. minimum* strains at different growth phases. The dynamics of DLI was followed during successive growth phases under controlled conditions and expressed in dependence on culture density and carbon content (calculated from cell volumes). DL decay kinetics was measured after white light illumination over 1–60 s.

2. Materials and methods

2.1. Algal cultures

The Baltic strain of *P. minimum* (BAL) was collected in the Baltic Sea and isolated at the Kristineberg Marine Research Station, Sweden. The American *P. minimum* (D5) was obtained from D. Stoecker, originally isolated from Chesapeake Bay, USA. The Adriatic *P. minimum* (PmK) was isolated from the Gulf of Trieste, Italy.

The stock cultures were maintained at the Laboratory of Marine Biology, Trieste, at 15 °C,

under cool-white fluorescent light (14:10 h light:dark cycle, average 70 μmol m^{-2} s^{-1} PAR). They were cultured in f/2-Si medium (Guillard, 1975) at salinities of 32 for PmK, 16 for D5, and 8 for BAL. These salinities correspond to the salinities found at the place of origin of the different strains.

For the growth experiment, each *P. minimum* strain was cultured in three 500 ml Erlenmeyer flasks containing 200 ml of medium at 20.5 ± 0.5 °C, under cool-white fluorescent light (12:12 h light:dark cycle, average 50 μmol m^{-2} s^{-1} PAR). The initial cell concentration approximated 1400 cells ml^{-1}.

2.2. Algal analyses

Samples for the cell count measurements were preserved with 2% formaldehyde (final concentration). They were examined using an inverted microscope (Utermöhl, 1958) and a Coulter Multisizer electronic particle counter (100 μm orifice tube with a dimensional range of 2–40 μm). In the Coulter counter each sample was counted twice and diluted 1:20 to keep the coincidence error under 10%. The curves obtained by the Coulter counter were processed using the Acucomp® software.

Cell volumes were estimated based on the total volumes given by the Coulter counter. The resulting volumes were converted into organic carbon values using the conversion factor: pgC = cell volume (μm^3) × 0.13 (Edler, 1979).

Cell dimensions and concentrations were determined by the Coulter counter on the same samples as used for DL measurements. To obtain cell length and width, and to confirm the volumes and numbers data from the Coulter counter, the cultures were examined under the microscope on days 1, 7, 13, and 23.

Samples for chlorophyll *a* analysis were taken four times (on days 1, 7, 13 and 23), filtered through a glass microfiber Whatman GF/C filter (Whatman Ltd., Maidstone, England), and extracted with methanol and pigment determined according to Vollenweider (1974).

2.3. Delayed luminescence measurement

DL was measured in a custom-made photon-counting luminometer. The apparatus included an illuminator for photo-excitation, a thermostated sample holder, and a light detector. A 20 W halogen lamp was used as the illuminator, providing light intensity of 40 μmol m^{-2} s^{-1} PAR at the sample position. The sample holder for 5 ml cuvettes was temperature-controlled to 21 ± 0.1 °C. For light detection, a red-light-sensitive photomultiplier tube (Hamamatsu R1104) with a Hamamatsu C3866 Photon Counting Unit for signal conditioning and amplification was used, coupled to a personal computer for data collection. Background noise (dark current) was measured prior to each DL measurement.

DL measurements were performed every second day until day 17 and then every third day. For each measurement, 2 ml of algal culture was transferred to a cuvette and dark adapted for 10 min. The cuvette was then inserted into the luminometer and illuminated for 3 s. The DL decay curve was measured in the interval 1–60 s after the illumination pulse with a 0.1 s integration time. The sample was then fixed with 0.1 ml of 40% formaldehyde for subsequent cell counting.

Average background noise was subtracted from all luminescence data. DLI was calculated as the sum of measured photon counts between 1.1 and 2.1 s after sample illumination. The possible effect of self-absorption of light with increasing culture densities was checked by measuring DLI of a dilution series.

3. Results

3.1. Culture growth of P. minimum strains

The three strains differed in cell size and chlorophyll *a* content. The Adriatic *P. minimum* (PmK) had the largest cell dimensions with an average length of 17.0 ± 1.2 μm. The Baltic strain (BAL) had the lowest average length (11.6 ± 1.7 μm) and the American strain (D5) was intermediate (14.0 ± 1.4 μm). The PmK and D5 strains decreased in cell dimension after day 23, while BAL did not markedly reduce its cell size until the end of the experiment. The PmK strain had the highest average cell chlorophyll *a* content (10–25 pg $cell^{-1}$), D5 had intermediate values (5–10 pg $cell^{-1}$), and the BAL strain had the lowest per cell (2–5 pg $cell^{-1}$).

All three strains had a lag phase from day 1 to day 7 (Fig. 1). The PmK and D5 strains were then in the

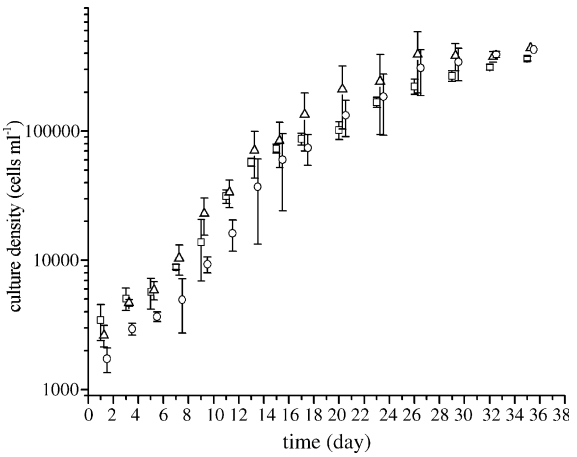

Fig. 1. Growth of three cultured *P. minimum* strains. Data from three replicates of each strain are presented (mean and standard deviation): (□) BAL, (△) D5, (○) PmK. Data points from D5 and PmK are shifted to the right by 0.25 and 0.5 day, respectively, to improve image clarity.

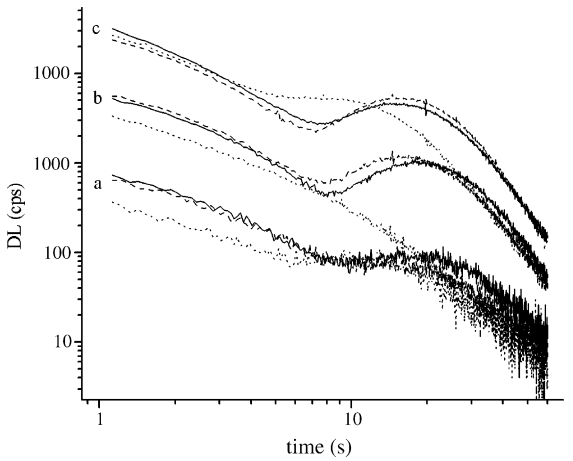

Fig. 2. Delayed luminescence decay curves of three different strains of *P. minimum* on days 5 (a), 13 (b), and 32 (c), (···) BAL, (- - -) D5, (—) PmK, cps: counts per second.

exponential phase until days 23–26, with similar cell densities (200,000 to 300,000 cell ml^{-1} on day 23). Declining and stationary phases followed with final culture densities just under 400,000 cells ml^{-1} (day 35). The BAL strain remained longer in the exponential phase and it reached lower final culture densities. The growth rate of PmK and BAL strains doubled at the onset of the exponential phase, and increased by 50% in strain D5. Senescence was observed in two replicates of D5 strain after reaching approximately 350,000 cells ml^{-1}; therefore, only data below this cell density was used for DLI monitoring in all strains.

3.2. Delayed luminescence

DL decay kinetics of *P. minimum* was, in general, hyperbolic in the first 5–6 s, followed by a distinct peak (Fig. 2). The onset of the peak in the BAL strain was earlier than in the other two strains and was less pronounced and not present at all between days 10 and 19. The DL decay curves from strains PmK and D5 showed more similarities. The peak became more pronounced with culture growth and reached a maximum earlier in both strains.

The DLI increased with culture density and carbon content. The calculated carbon content reached approximately 30 μg ml^{-1} at culture densities of 350,000 cells ml^{-1}. DLI versus cell concentration was linear at the observed culture densities.

The PmK strain had the highest average DLI per cell values, D5 intermediate and BAL the lowest values (Fig. 3a). There was an increase in DLI per cell during the lag phase in strains PmK and BAL, which was less pronounced in D5. Then the DLI per cell in PmK progressively decreased. DLI cell^{-1} remained constant in D5 and remained stable in BAL after it peaked on day 9.

The carbon content per cell (Fig. 3b) reached the highest values for the PmK strain (in the range of 0.25–0.30 ng cell^{-1}) and lowest for BAL (0.15 ng cell^{-1} on average). The three strains showed different temporal patterns of carbon per cell. There was a general decline in PmK. In D5, the decline started after two weeks, while in BAL, carbon increased during the lag phase, decreased for five days, and increased during the second part of exponential growth.

DLI increased in a two-stage process in the first two growth phases, while there was no evident pattern in the declining phase. Therefore, only the data of the first two phases could be pooled in Fig. 4a and b. The steeper first part, corresponding to the lag phase, was from the beginning to cell density approximately 10,000 cells ml^{-1} or 2 μg carbon ml^{-1}, followed by the second, less steep portion, corresponding to

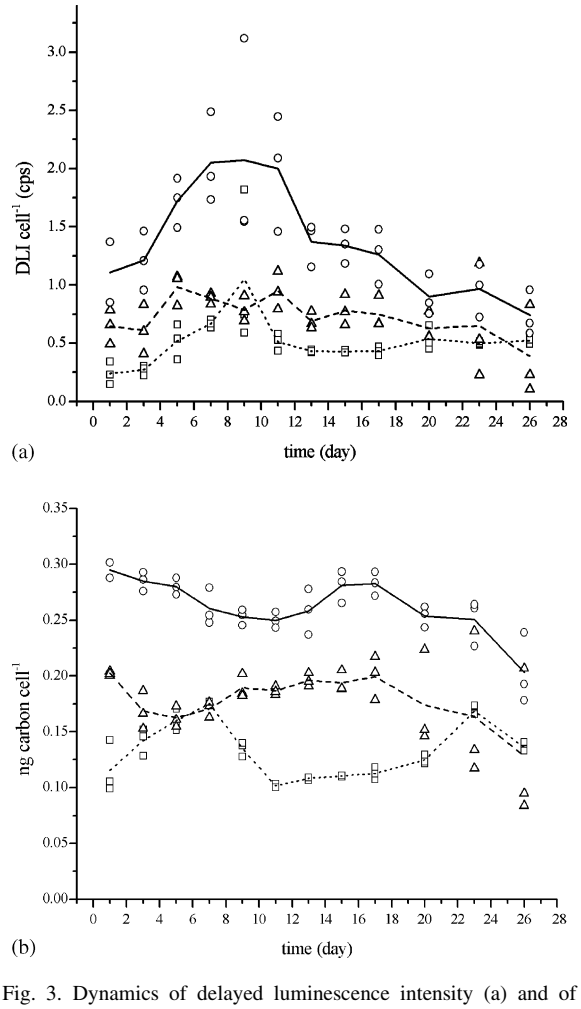

Fig. 3. Dynamics of delayed luminescence intensity (a) and of carbon content (b) calculated per single cell and in three *P. minimum* strains: (□) BAL, (△) D5, (○) PmK. The lines represent averages for three replicate cultures of each strain: (···) BAL, (- - -) D5, (—) PmK, cps: counts per second.

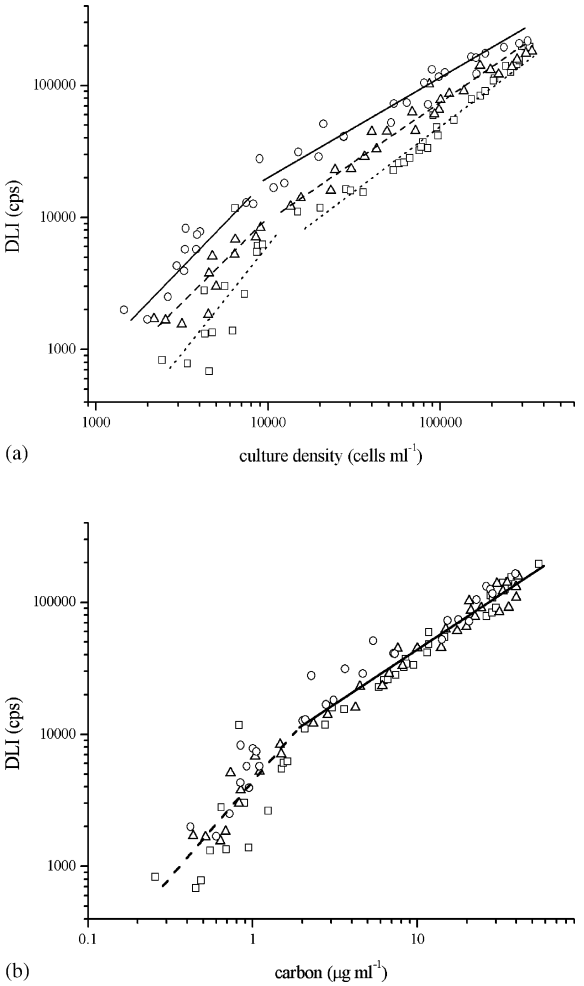

Fig. 4. Delayed luminescence intensity of three *P. minimum* strains expressed as a function of culture density (a) and carbon content (b): (□) BAL, (△) D5, (○) PmK, cps: counts per second. To quantify the two observed phases we calculated the linear regression of the pooled and log–log transformed data. The regression curves are given by $\log(\text{DLI}) = A + B \times \log(\text{culture density})$ (a) and $\log(\text{DLI}) = A + B \times \log(\text{carbon content})$ (b); R is the correlation coefficient. (a) The data are pooled for each strain: (···) BAL, (- - -) D5, (—) PmK. Regression parameters: BAL: <10.000 cells ml^{-1}: $A = -3.23 \pm 1.66$, $B = 1.77 \pm 0.44$, $R = 0.77$; >10.000 cells ml^{-1}: $A = -0.19 \pm 0.18$, $B = 0.98 \pm 0.07$, $R = 0.98$; D5: <10.000 cells ml^{-1}: $A = -1.12 \pm 0.74$, $B = 1.27 \pm 0.20$, $R = 0.90$; >10.000 cells ml^{-1}: $A = 0.63 \pm 0.18$, $B = 0.84 \pm 0.04$, $R = 0.98$; PmK: <10.000 cells ml^{-1}: $A = -1.23 \pm 0.58$, $B = 1.40 \pm 0.16$, $R = 0.93$; >10.000 cells ml^{-1}: $A = 1.35 \pm 0.19$, $B = 0.73 \pm 0.04$, $R = 0.97$. (b) The data are pooled for all strains: (- - -) lag phase, (—) exponential growth. Regression parameters: <2 μg ml^{-1}: $A = 3.63 \pm 0.04$, $B = 1.39 \pm 0.18$, $R = 0.80$; >2 μg ml^{-1}: $A = 3.83 \pm 0.03$, $B = 0.81 \pm 0.03$, $R = 0.96$.

exponential growth. The three strains had similar DLI versus culture density relationships, with the highest values for the PmK strain and the lowest for BAL.

The goodness-of-fit test confirmed that two regression lines describe the DLI versus culture density or carbon content better than only one line, with better fit in Fig. 4b (chi-square for one regression was 0.020 and for two lines 0.026). The comparably smaller variation of the data points in Fig. 4b results from accounting for the changes in cell size during culture growth when calculating carbon content.

4. Discussion

The growth curves were similar for the PmK and D5 strains, whereas the BAL strain had prolonged exponential growth compared to the other two strains. This might be caused by different salinity of the medium. Experiments at equal salinity are being planned to test this hypothesis. For this experiment, cultures were grown at salinities that matched those of the areas where the strains were collected. There was also a clear relation between the cell size and salinity of the medium. PmK, grown at 32 PSU, had the largest cells, whereas BAL, grown at 8 PSU, the smallest cells. Hulburt (1965) and Hajdu et al. (2000) reported a negative correlation between *P. minimum* cell size and salinity *in situ*, whereas other dinoflagellates showed a positive correlation (Plinski and Jozwiak, 1986). Different cell sizes also reflected the average DLI and chlorophyll *a* per cell values, as seen from our experiments.

PmK and D5 were in the retardation phase after day 23. The results showed that the decrease in the cell size at the end of culture growth was linked to the physiological state of the algae. Cell proliferation decreased, and therefore fewer cells were preparing for division by increasing their size. This decrease was lower in the BAL strain, because it only started its stationary phase of growth at the end of the experiment. The low DLI cell^{-1} and carbon cell^{-1} values of D5 on days 23 and 26 are probably due to cell senescence.

The three *P. minimum* strains differed in cell sizes, chlorophyll cell^{-1} content, and subsequently in their DLI cell^{-1}. These differences were not constant, but changed with culture growth. It was also possible to distinguish among the strains by comparing their DL decay kinetics. Again, more similarities were observed between the PmK and D5 strain. At this point, the comparison of DL decay curves is qualitative, because it was not possible to accurately calculate the peak position or peak surface area without knowing the exact time dependence of the monotonic portion of the relationship (e.g., the baseline) or without having a relevant model for the physiological processes underlying peak formation.

The presence of a peak in DL decay curves shows the involvement of PSI in generation of DL (Desai et al., 1983; Hideg et al., 1991). In our case, the peak could be the consequence of the far red light from the illuminating pulse (produced by a halogen lamp with maximum emission at wavelengths larger than 700 nm) better absorbed by PSI than PSII (Desai et al., 1983, Mellvig and Tillberg, 1986). Cyclic electron flow produced excess ATP over NADPH, which could generate back electron flow and a peak in DL kinetics (Mellvig and Tillberg, 1986). Quantification of DL decay curves by further studies is important for the development of a method for *in situ* bloom monitoring in complex marine environments. The combination of DL and fluorescence measurement is under study and could enable successful tracking of the bloom developmental state.

In this paper, the possible use of DL measurements for non-invasive monitoring of *P. minimum* growth was examined. It was possible to detect and follow two different phases of *P. minimum* growth under controlled growth conditions. The first, steeper, phase of the delayed luminescence response corresponded to the lag phase of the growth and the second phase to the exponential growth of the populations. The results show that DLI correlates better with carbon content than with culture density. DLI could therefore be used as an estimate of biomass irrespective of strain and for differentiating between lag and exponential phases of growth of monitored taxa.

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