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Nitrate reductase activity in the diatom *Biddulphia longicruris:* characterization and daily oscillation

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- *Abstract*: Nitrate reductase (NR) activity was studied in the marine diatom *Biddulphia longicruris*. During 24 hours of sampling, NR activity was found during day time and in the transition day-night. Nitrite anions, the product of nitrate reduction, was released by the cells at the times NR was active, and accumulated in the culture medium. Whenever the cultures of *B. longicruris* were submitted to nitrogen deprivation, NR activity could not be detected. *In vitro* determination of K_M values for NR using nitrate or NADH were respectively 50 μM and 80 μM. Temperature and pH dependence of NR activity were also determined for this organism.

- *Resumo*: A atividade de nitrato redutase (NR) foi estudada na diatomácea marinha *Biddulphia longicruris*. A NR é a enzima responsável pelo processo de assimilação de nitrato. O nitrato é reduzido no interior da célula a nitrito pela ação da NR. Esta enzima é normalmente oligomérica e utiliza o NADH como substrato doador de elétrons. Em alguns organismos também são encontradas NRs capazes de utilizarem o NADPH como doador eletrônico para a redução do nitrato a nitrito. Para a diatomácea *B. longicruris* foram apresentadas evidências de que a sua NR é específica para NADH e que a redução de nitrato em presença de NADPH não acontece. Determinações *in vitro* dos valores das constantes de Michaelis-Menten (K_M) usando nitrato e NADH como substratos, são respectivamente 50 μM e 80 μM. A temperatura ótima de reação enzimática e a sua dependência ao pH também foram estudadas. Cultivos de *B. longicruris* foram acompanhados por períodos de 24 horas e foi mostrado que a atividade de NR é encontrada em maiores níveis durante os períodos de transição de luz/escuro. Os aníons nitrito, produtos da redução de nitrato, são eliminados pelas células nos períodos de maior atividade de NR e se acumulam no meio de cultura. Células submetidas à ausência de nitrato apresentam uma repressão da expressão de NR, sendo ativadas quando pulsos de nitrato são fornecidos a estas culturas.

- *Abbreviations:* NR - nitrate reductase; NAD(P)H - β-nicotinamide adenine dinucleotide (phosphate), reduced form; EDTA - ethylenediaminetetraacetic acid; BSA bovine serum albumin.

- *Descriptors: Biddulphia longicruris,* Daily variations, Nitrate reductase, Nitrate uptake.

Introduction

Nitrogen metabolism in algal species has been extensively studied in order to relate nitrogen availability in the marine environment and algal population growth rates (Dortch & Postel, 1989).

Nitrate sustains gross primary productivity and there is a considerable amount of it being transported from the deeper layers, by physical processes like vertical mixing or upwelling (Smetacek & Pollehne, 1986). After being taken up by the cells, nitrate can either form an intracellular pool (Collos, 1982) or immediately undergo the metabolic reduction steps. Nitrate reductase (NR), the first enzyme

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of the nitrate assimilatory pathway, catalyzes the reduction of nitrate to nitrite.

Previous studies have established that NR exhibits a diurnal variation in its expression and some hypotheses have been proposed for the possible control mechanisms. In natural populations, the circadian expression of this enzyme was reported by Eppley *et al.* (1970) in the Peruvian current. The photosynthetic dinoflagellate *Gonyaulax polyedra* displays a circadian expression of NR controlled by the biological clock (Ramalho *et al.*, 1995). Velasco *et al.* (1989) showed that daily variation in NR activity of *Chlorella sorokiniana* is due to "de novo" biosynthesis of the apoprotein during the light period followed by degradation at the dark period.

Nitrogen-assimilating organisms, such as diatoms, incorporate inorganic nitrogen to organic macromolecules in the ocean. Being at the base of the food chain, they sustain the whole cycling of energy and matter in the marine environment. Therefore, studies on the nitrogen assimilating enzymes are the major importance in the undestanding the process and mechanisms of nitrogen metabolism. We have evaluated the relative importance of the light-dark cycles and nitrate availability in nitrogen-deficient cultures with respect to the daily variations in nitrate assimilation of the diatom *Biddulphia longicruris*. Our studies have further shown a novel behavior of nitrogen assimilation: a semi-diurnal oscillation of NR activity in batch cultures of these cells.

Material and methods

Cell Culture: The diatom *Biddulphia longicruris* was isolated from the estuarine waters of Cananéia, on the southeastern Brazilian coast (25°S, 048°W). Cultures of *B. longicruris* were grown in enriched sea water, f/2 culture medium (Guillard & Ryther, 1962) at 20° ± 1°C, under alternating periods of 12 hr light (cool white fluorescent light, 160 $mol.m^{-2}.s^{-1}$) and 12 hr dark to a cell density of about 10^4 cells/mL. The number of cells was determined in Nageotte chamber, under light microscope observation.

Assay of Nitrate Reductase: Cell extracts were prepared by harvesting 1.5 liters of cell culture at the beginning of the light period. Freshly harvested cells were resuspended in 4 mL extraction buffer (0.05M Tris-HCl buffer pH 7.9 containing 0.05 M sucrose, 0.01 M EDTA and 5 mM 2-mercaptoethanol) and disrupted by high pressure of N_2 (2,000 psi), in a Nitrogen Pump (Parr Instrument Company). Cell debris was removed by centrifugation at 10,000 x g for 20 minutes. The reaction mixture for NR assays contained 0.5 mL of the cell extract in 1.3 mL of 0.2 M phosphate buffer pH 7.0, 14 mM KNO_3, 210 μM $MgSO_4$, 80 μM NAD(P)H (Harrison, 1973). NAD(P)H was added at zero time and the mixture was incubated at 25°C. Control suspensions were incubated in the absence of NAD(P)H. The reaction was stopped by placing the reaction tubes in a boiling water bath. Nitrite concentration was determined by adding 40 mM sulfanilamide solution followed by 2 mM n-(1-naphthyl) ethylenodiamide dihydrochloride solution (Grasshoff *et al.*, 1983). The red color (measured at 543 nm) which develops after 10 minutes is proportional to nitrite concentration.

In order to estimate the Michaelis-Menten constant (K_M) for both nitrate and NADH the phosphate buffer was used at the same conditions except for the substrate concentration. Experiments carried out to determine the NADH K_M, 14 mM KNO_3 was used and, the concentration of NADH varied at the range of 0 to 350 μM. Experiments performed in order to estimate the nitrate K_M, 80 μM NADH was used and, the concentration of nitrate varied at the range of 0 to 500 μM.

One unit of NR activity is defined as the amount of enzyme required to produce 1 μmol of nitrite/minute at 25°C. Protein content in the crude extract was estimated by Coomassie blue dye binding assay (Bradford, 1976). BSA (Sigma) was used as the standard protein for calibration curves.

Nutritional and photo-period pre-conditionings: The concentration of nitrate, nitrite and NR activity were measured for populations grown under three different culture conditions: batch culture and nitrate deficient cultures where nitrate was added either at the beginning of the light period or at the beginning of the dark period. The nitrogen-limited cell cultures were obtained by growing diatoms receiving a daily pulse of nitrate (10 μM) always at the same time. We have previously observed that this concentration of nitrate is totally consumed over a LD cycle.

Cellular uptake of nitrate: Two aliquots were taken from dense culture flasks every 2 hours for over a 24 hours period, under the culture conditions described above. Nitrite ion content from the cell-free medium was estimated by a colorimetric method. For nitrate measurements, it was first chemically reduced to nitrite in a copper-coated cadmium column and the difference between the concentration of nitrite after the reduction and the former nitrite concentration in the cell-free culture medium results in the nitrate content (Grasshoff *et al.*, 1983).

Results

As for many other nitrate reductases, such as from *Arabidopsis thaliana, Neurospora crassa*, tobacco and tomato (see Adam & Mortenson, 1985), NR in *B. longicruris* is active at neutral pH (Fig. 1). As higher

plants and other microorganisms, *B. longicruris* NR is stable over a wide temperature range (4 - 35°C).

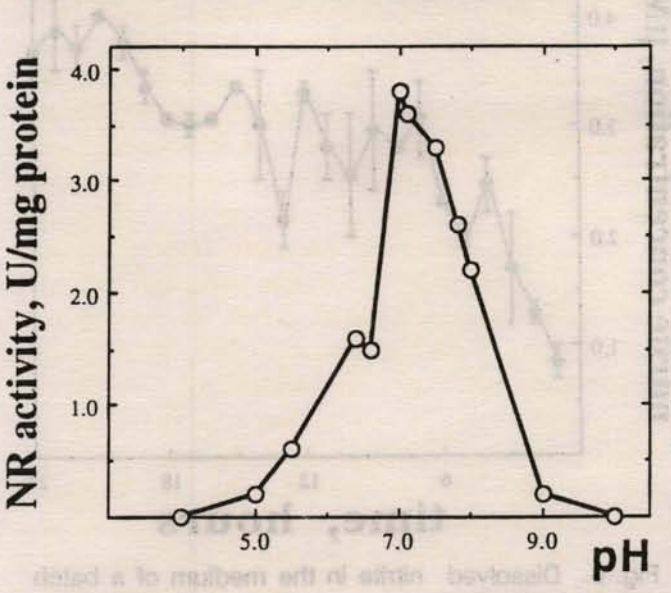

Fig. 1. pH dependence of *B. longicruris* NR. The activity of NR from crude extract was estimated as described in Material and methods.

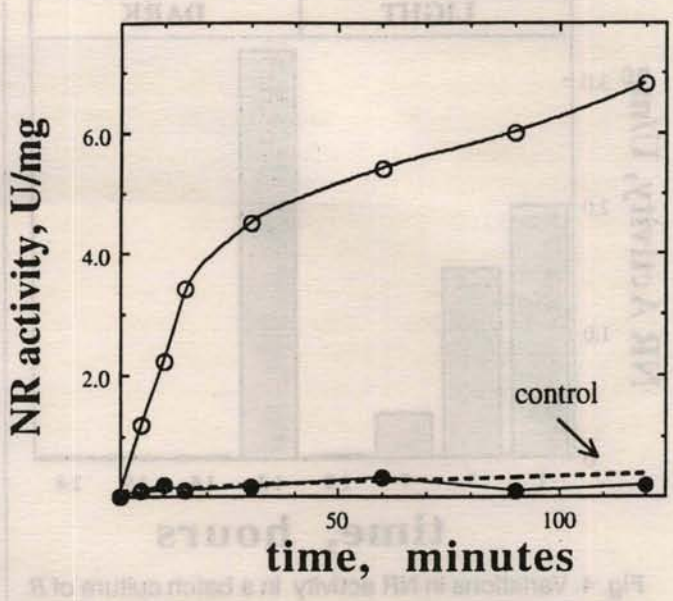

Fig. 2. Electron donnor of *B. longicruris* NR. Crude extracts of *B. longicruris* were incubated at the same conditions with either NADH (-o-) or NADPH (-●-) and the activity was measured by the production of nitrite. Control (---) has no addition of NAD(P)H.

As shown in the Figure 2 it was not found NR activity when NADPH was used as an electron donor to the reduction of nitrate. *In vitro* estimation of K_M values for NR using nitrate or NADH were respectively 50 μM (Fig. 3A) and 80 μM (Fig. 3B). The assays were performed in 0.2 M phosphate buffer, pH 7.7, at 25°C. The enzyme has about the same affinity for nitrate and for NADH, as do many other NRs previously described.

For the batch cultures, NR activity was observed in two restricted periods of the day, at the beginning of the light or the dark periods (Fig. 4). At the beginning of the light period the NR activity was found to be 2.0 U/mg decreasing about 25% after 3 hours of light exposure. After 7 hours of light exposure the cells contain only 20% of the initial activity reaching no activity 3 hours latter. The cellular NR activity is recovered at the beginning of the dark period.

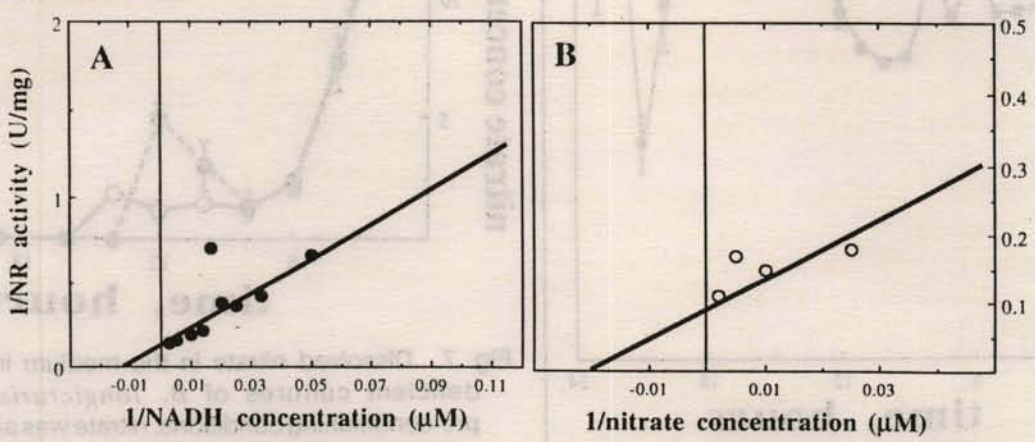

Fig. 3. *In vitro* determination of K_M values for NR. Crude extracts of *B. longicruris* were incubated with different concentrations of NADH (A) and different concentrations of nitrate (B), as described in Material and methods. The activity of NR from crude extract was estimated as described above.

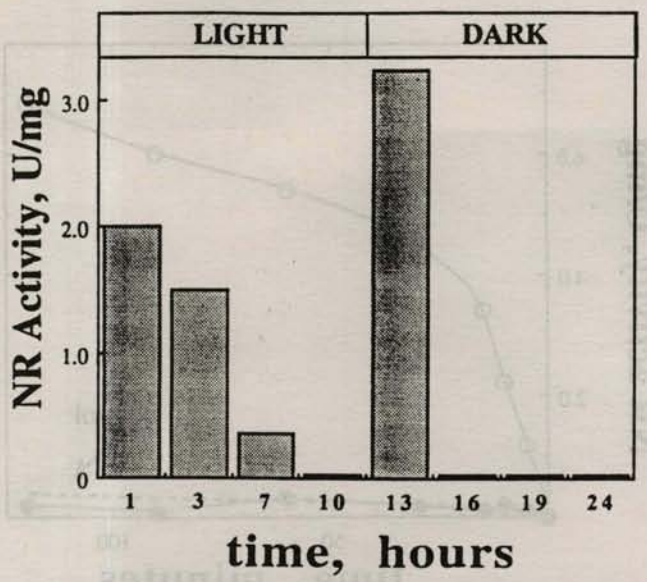

Fig. 4. Variations in NR activity in a batch culture of *B. longicruris*, grown under light-dark cycle conditions. Similar pattern was obtained in three different sets of culture flasks.

Figure 5 displays the variation of nitrate dissolved in the culture medium measured by the presence of nitrite after medium samples being chemically reduced by copper-coated cadmium column. As can be noticed the nitrate concentration varies significantly over 24 hours showing a high uptake at the end of the light period and at the end of dark period. A similar pattern of uptake/excretion of nitrate was recently reported by Lópes- Figueroa & Rüdiger (1991). Dissolved nitrite in the medium rose from 0.5 to 4.0 μM (Fig. 6).

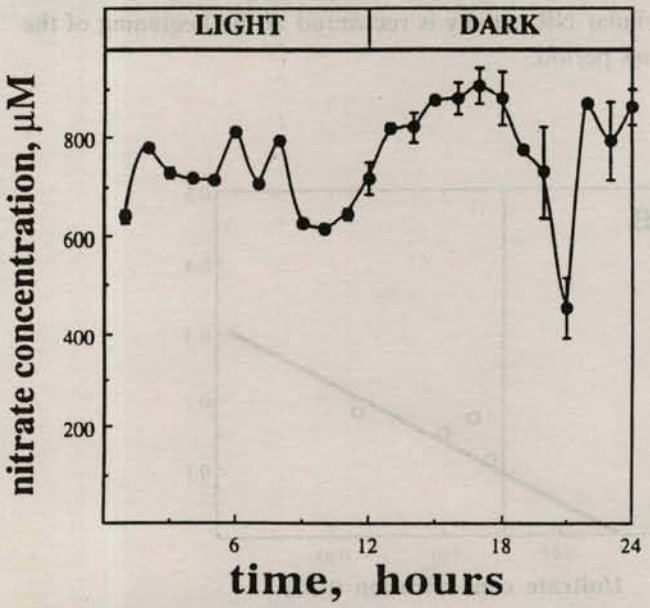

Fig. 5. Dissolved nitrate in the medium of a batch culture of *B. longicruris*, grown under light-dark cycle conditions. The standard deviations calculated are represented by the vertical lines, and points represent medium values.

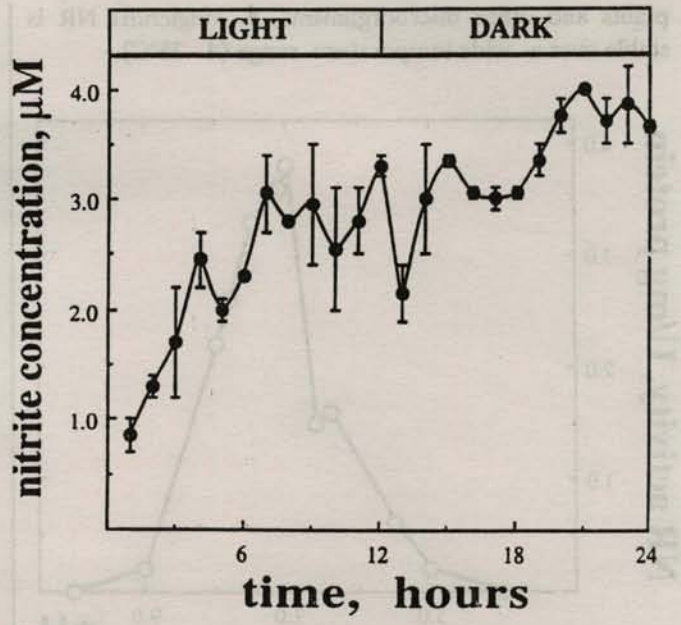

Fig. 6. Dissolved nitrite in the medium of a batch culture of *B. longicruris*, grown under light-dark cycle conditions. The standard deviations calculated are represented by the vertical lines and points represent medium values.

For the N-limited cultures, the two nitrogen deficient cultures grown under light-dark cycle condition to which nitrate had been given at different times of the day show no differences in the ability to take up nitrate at the beginning of the light period (Fig. 7). This result suggests

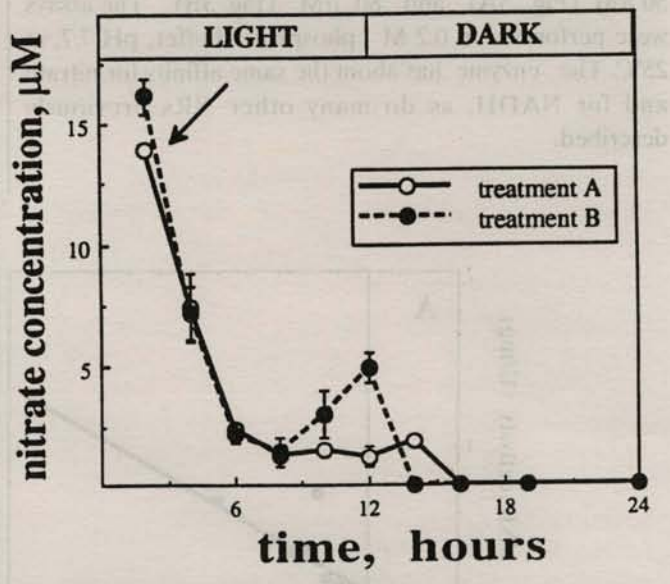

Fig. 7. Dissolved nitrate in the medium in nitrogen deficient cultures of *B. longicruris*. In the pre-conditioning conditions, nitrate was added daily as a 10 μM pulse at the beginning of the light cycle (treatment A) or at the beginning of the dark cycle (treatment B). Arrow indicates the nitrate pulse added at the experiment. The standard deviations calculated are represented by the vertical lines and points represent medium values.

that nitrogen deficient cultures, grown under light-dark cycle conditions are able to utilize nitrate from the culture medium as soon as it is available.

Nitrogen deficient culture conditions express NR activity at basal levels that cannot be measured at micromolar levels. Since nitrate was the only source of nitrogen, activity of this enzyme could not be used to estimate the nitrogen status of this algal population.

Discussion

The major source of nitrogen in the marine ecosystem is in the form of nitrate, which must be reduced to ammonia or amine to be used by cells in the biosynthesis of nitrogen-containing macromolecules such as protein and nucleic acids. The reduction of nitrate to nitrite, the first step, is catalyzed by a NADH-dependent NR and may be rate-limiting in the nitrate assimilation process. NR is typically a high molecular weight flavoprotein that uses NADH or NADPH as an electron donor and contains two enzymatic centers which participate sequentially in the reduction of nitrate to nitrite. Nitrite is then reduced to ammonia by nitrite reductase.

Although the overall enzymatic functions of NRs from various different eukariotes are similar, and they are typically oligomeric enzymes, the number, sizes, and types of subunits can vary considerably (Ramalho *et al.*, 1995). The biochemical properties of *B. longicruris* NR were found to be somewhat different from those previously described (Adam & Mortenson, 1985). Although the apparent K_M values were close to those described for NR in the chlorococcale *Ankistrodesmus braunii* (de la Rosa *et al.*, 1981), and the fungus *Neurospora crassa* (Horner, 1983), *B. longicruris* NR has a much lower affinity for nitrate than that found in the marine dinoflagellate *Gonyaulax polyedra* (Ramalho *et al.*, 1995). However, it is interesting to note the differences between the enzymes of *B. longicruris, Ankistrodesmus braunii, G. polyedra* and *Chlorella vulgaris*. Aside from the subunit structural differences, *A. braunii* and *G. polyedra* use either NADPH or NADH, whereas *B. longicruris* is specific for NADH, similar to *C. vulgaris* NR.

The wide temperature range found for *B. longicruris* NR may be related to an advantage for this organism indicating that such cells are able to adapt themselves to the marine water temperature variation. Other marine microorganisms have similar behavior for NR activity (Ramalho *et al.*, 1995).

The senoidal pattern observed in nitrate dissolved in the medium (Fig. 5) can be regarded either as a simple phenomenon of adsorption/release by the plasma membrane or, as nitrate uptake followed by excretion. The decrease of nitrate concentration in culture media is assumed to be caused by the uptake, whereas the rise in the extracellular nitrite concentration is caused by the excretion. By the methods used we are not able to discriminate between these different processes since the intracellular pool of nitrate was not measured. Recently Lópes-Figueroa & Rüdiger (1991) have reported a similar response for nitrate behavior when *Ulva rigida* was studied. Studies on rates of nitrate uptake in *Skeletonema costatum* (Serra *et al.*, 1978 a, b) showed that this process fits in the Michaelis-Menten model only for low nitrate concentrations. For *S. costatum*, there are two simultaneous processes acting on the membrane transport: simple diffusion, and a permease dependent transport which saturates at 6 μM.

As can be noticed at Figures 3 and 4, a time shift in nitrate uptake and NR activity can also be observed. A similar temporal shift has been observed previously by Dortch *et al.* (1979) working with *S. costatum*. *S. costatum* is able to store nitrate as an intracellular pool until nitrate from the environment has been completely exhausted. Then, the intracellular pool is enzymaticaly reduced to nitrite. A semi-diurnal rhythmicity in these processes was evident only in batch cultures grown under light-dark cycle conditions. Since *B. longicruris* NR activity was not detected in batch cultures grown under constant light conditions (data not shown), the semi-diurnal pattern in nitrate uptake, observed in light-dark conditions, is possibly as a consequence of the NR activity.

By the results presented in this paper, the diatom *B. longicruris* is able to reduce nitrate mainly at the beginning of light period and at the beginning of dark period. Nitrate uptake is more prominent in both, at the end of light period and the end of dark period, a few hours in advance of the time which NR is active. However, cellular nitrite release increases gradually during the 24 hours experiment in a much lower concentration than nitrate uptake.

Acknowledgments

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