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Optimized nitrate reductase assay predicts the rate of nitrate utilization in the halotolerant microalga *Dunaliella viridis*

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Abstract

An *in situ* method for measuring nitrate reductase (NR) activity in *Dunaliella viridis* was optimized in terms of incubation time, concentration of KNO_3, permeabilisers (1-propanol and toluene), pH, salinity, and reducing power (glucose and NADH). NR activity was measured by following nitrite production and was best assayed with 50 mM KNO_3, 1.2 mM NADH, 5% 1-propanol (v/v), at pH 8.5. The estimated half-saturation constant (K_s) for KNO_3 was 5 mM. Glucose had no effect as external reducing power source, and NADH concentrations >1.2 mM inhibited NR activity. Nitrite production was linear up to 20 min; longer incubation did not lead to higher nitrate reduction. The use of the optimized assay predicted the rate of NO_3^- removal from the external medium by *D. viridis* with high degree of precision.

Introduction

Nitrogen assimilation – from external nitrogen to amino acids – by phytoplankton depends on the integrated activity of a number of enzymes; the first step in nitrate assimilation is its reduction to nitrite by means of nitrate reductase (NR); then, nitrite reductase reduces nitrite to ammonium and the glutamine synthetase/glutamine- oxoglutarate aminotransferase (GS/GOGAT) system completes major nitrogen assimilation (Syrett, 1981).

NR activity is usually considered to be either the rate-limiting for nitrogen assimilation or closely coupled to the rate-limiting step (Eppley et al., 1970; Berges & Harrison, 1995a). If this is true, NR activity measurements would be able to predict the rate of external nitrate assimilation to organic material if nitrate being derived for intracellular storage is known; indeed, NR activity has been widely used by marine biologists as an indicator of nitrate utilization by phytoplankton (Eppley et al., 1970; Blasco & Packard, 1974; Blasco et al., 1984). Traditionally the method used has been the *in vitro* assay developed by Eppley et al. (1969) and improved by Eppley (1978) and Packard et al. (1978). The main problem of the *in vitro* assay is that sometimes the enzyme acitivity can not be detected, indicating that it has been degraded or inactivated. Nevertheless, recently several authors have improved the *in vitro* method for estimation of NR activity in several marine micro and macroalgae (Berges & Harrison, 1995a, b; Hurd et al., 1995). On the other hand, *in situ* measurements are an alternative method when *in vitro* problems cannot be overcome, and give higher NR activities in some cases (Hochman et al., 1986; Hurd et al., 1995).

The *in situ* method requires: (i) buffer, (ii) compound able to permeabilise the membrane to substrates and products, (iii) source of NO_3^- and (iv) source of reducing power. Production of nitrite is measured. This assay must be performed in the dark to avoid the further reduction of NO_2^- by nitrite reductase which needs reduced ferredoxin from light reactions. It also has to be done under anaerobic conditions to avoid the competition between nitrate and O_2 for the NADH (Canvin & Woo, 1979). The term *in situ* is used to stress that the enzymatic activity is being assayed at its cellular

site. It is common to find in the literature the same sort of technique referred to as *in vivo* NR assay; however, Corzo and Niell (1991) proposed that *in vivo* should be reserved for non-intrusive methods, in which the biological material could grow again after the assay. Many variations exist in the *in situ* method depending on the species and on the preferences of the investigators for one compound or another, but it must be pointed out that NR activity has different behaviour depending on the species, assay conditions and reaction mixture composition (Hochman et al., 1986).

Hypersaline environments are distributed all over the planet, being specially important in arid and semiarid areas. Among the organisms that inhabit these continental water bodies, *Dunaliella* is the best known and possibly is ubiquituous in hypersaline systems (Borowitzka & Borowitzka, 1988; Javor, 1989). Species of the genus *Dunaliella* are the only eukaryotic microorganisms that can survive in saturated salt solutions (>5 M NaCl) (Javor, 1989). It is an obligately phototrophic, oxygenic, aerobic unicellular organism, representing the most important primary producer in continental saline waters (Javor, 1989). *Dunaliella* species show high requirements of nitrate for growth, and most of the authors propose an optimum nitrate concentration of 5–10 mM (Johnson et al., 1968; Borowitzka & Borowitzka, 1988) to achieve optimal yield.

As NR activity differs between species and there are no current data for halotolerant microalgae, the aim of this study is to set the optimal conditions for the *in situ* nitrate reductase assay to enable an accurate prediction of NO_3^- utilization in the halotolerant microalgae *Dunaliella viridis* Teodoresco.

Material and methods

Dunaliella viridis was isolated from the athalassic lake of Fuente de Piedra (37°06′ N, 04°45′ W). It was grown in batch cultures in the medium of Johnson et al. (1968) at 1 M NaCl and 5 mM NO_3^-, without TRIS buffer, under continuous white light (250 μmol m^{-2} s^{-1}) provided by Silvania Daylight (20 W) commercial fluorescent lamps, at 25 °C in continuous orbital shaking.

The *in situ* NR assay was performed by taking 1 ml of cell suspension (5–30 10^6 cell ml^{-1}) in NO_2^--free medium and adding 2 mL of reaction mixture. This initial mixture consisted of 0.1 M phosphate buffer, 0.5 mM EDTA, 5% (v/v) 1-propanol, 30 mM KNO_3, 1 M NaCl, pH 8.0. Before the addition of the cells, the reaction tubes and the cell suspension were separately bubbled for 2 min with N_2 in order to remove oxygen. The tubes were quickly sealed and covered with aluminium foil and so incubated in the dark at 30 °C in a water bath under continuous shaking (50 rpm). Other parameters of the assay were changed and tested. Incubation time, NO_3^- concentration, pH, salinity, substitution of NaCl by glycerol in the assay medium, permeabilising agents (propanol and toluene) and reducing power (glucose and NADH) were tested. The concentration of EDTA (0.5 mM) and phosphate buffer (0.1 M) were selected following the work of Corzo and Niell (1991) for the green alga *Ulva rigida*; pH of the reaction mixture was monitored and did not significantly deviate from the test values during the incubation period.

After the incubation, the reaction was stopped by filtering the assay mixture (Whatman GF/F) in order to remove the enzyme-containing cells. The filtered solution was used for nitrite determination according to Snell and Snell (1949). Nitrite concentration remained constant once the medium was filtered indicating that no significant active enzyme from broken or damaged cells passed through the filter. When NADH was present in the reaction mixture, it had to be removed before the diazotization reaction to allow color evolution. Active charcoal (2.78 mg mL^{-1}) was used to remove the remaining NADH added in the solution by adsorption (after strong stirring) and centrifugation (Hernandez et al., 1993). This procedure reduced completely the absorbance in the 340 nm region ensuring a total removal of interfering NADH.

NR measurements under the optimal assay conditions found were compared with real NO_3^- uptake rates. For this comparison, *D. viridis* was grown as described above; exponentially growing (N-replete) cells were centrifuged and resuspended in fresh medium with 1 mM KNO_3. NO_3^- uptake was measured as NO_3^- disappearance from the culture medium in light during 8 h (sampling each 1 h) and NR activity was measured at 0, 4 and 8 h. External KNO_3 concentration was selected to be 1 mM at the beginning of the experiment rather than the 5 mM used in the growth medium to enable the detection of changes in NO_3^- in a time scale of few hours. Since NR activity is a potential measurement, with substrate being in saturation (50 mM), the obtained value was recalculated from the kinetic constants estimated in this work to obtain the activity corresponding to 1 mM.

NO_3^- concentrations were estimated using a Bran+Luebbe Technicon Traacs 800 flow analyser,

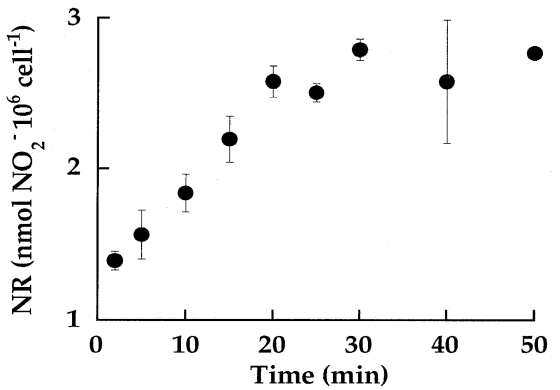

Figure 1. Time course of nitrite production during the NR assay.

using the methods of Shinn (1941) and Wood et al. (1967).

All cultures were run in duplicate and three determinations were performed from each culture; results were compared by means of a oneway analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$.

Results

Incubation time

The production of nitrite during the NR assay followed a linear trend during the first 20 min (Figure 1). After this period of time, nitrite production rapidly stopped, the concentration of nitrite in the medium remaining constant over the next 30 min. The limit of colour detection of the method to estimate nitrite was not achieved during this period so the non-linearity of the assay after 20 min was not due to oversaturation of colour detection by the method. The optimal incubation time was, therefore, stated in 20 min; longer incubation times might lead to underestimation of the nitrite production rate in *D. viridis*.

KNO_3 concentration

NR activity followed a hyperbolic saturation curve with increasing NO_3^- concentration in the assay medium (Figure 2a), indicating a typical Michaelis-Menten behaviour. The Lineweaver-Burk plot (Figure 2b) gave a half-saturation constant K_s of 5 mM and a maximum velocity (V_m) value of 11.2 nmol NO_2^- 10^6 cell^{-1} h^{-1}. The concentration of NO_3^- in the assay medium was never limiting (not shown), so the non-linearity of the

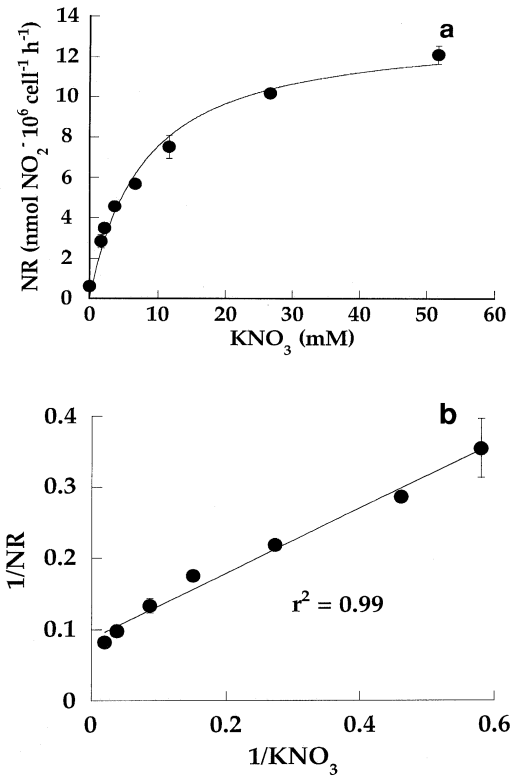

Figure 2. (a) NR activity in *D. viridis* as a function of nitrate concentration in the assay mixture; (b)Lineweaver-Burk plot.

assay time course after 20 min was not due to depletion of the substrate for the reaction.

Permeabilisers

Increasing concentration of propanol and toluene, both separately and mixed together, were tested. The highest NR activity was found with 5% (v/v) 1-propanol in the absence of toluene; increasing alcohol concentration led to a decrease of the NR activity (Figure 3). Toluene only gave higher activity than propanol when the concentration of the latter was below 3% (v/v). Toluene did not increase NR activity but it also caused the opposite effect, decreasing the effect of propanol when the latter was present in a concentration higher than 1% (v/v).

Salinity and glycerol

In the *in situ* NR assay, the outer membrane of the cells is permeabilised and the enzyme could be affected by the external medium. We tested the effect of increasing

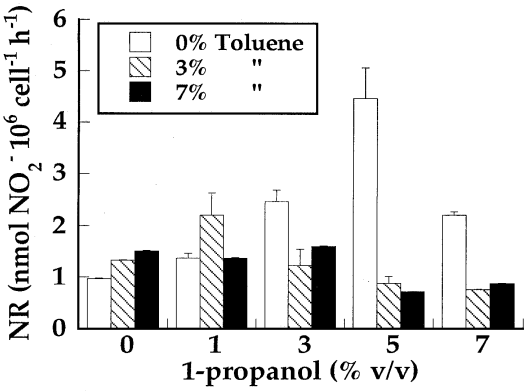

Figure 3. Effect of several toluene and propanol concentrations on NR activity in *D. viridis*.

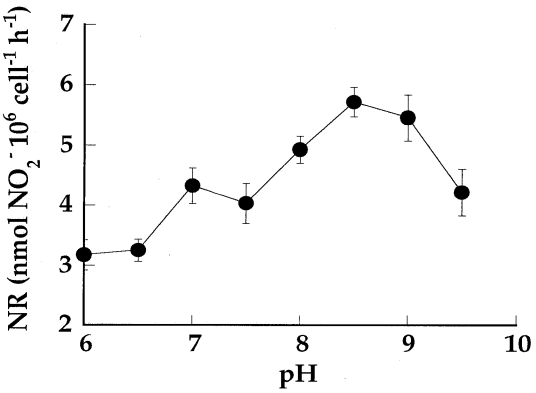

Figure 5. Variation of the NR activity as a function of external pH in the assay medium.

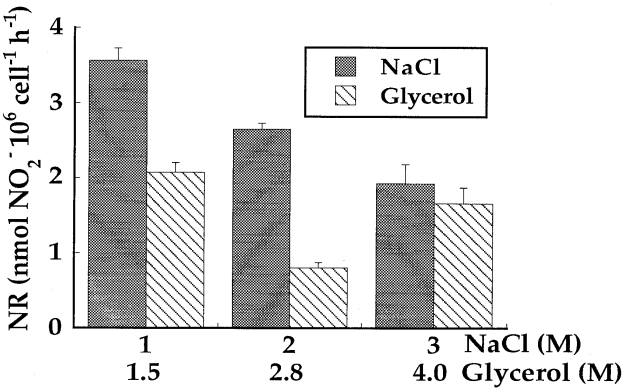

Figure 4. Influence of osmotic pressure in NR activity in *D. viridis*. Algae were grown at three different NaCl concentrations, and equivalent osmotic pressures in the reaction mixture were achieved by adding NaCl or glycerol.

salinity in the range 1–3 M NaCl, both in the culture and in the assay reaction mixture, and the substitution of this salt in the assay medium by glycerol, the natural osmotic compound produced by *D. viridis*. When glycerol was the osmotic compound present in the assay medium, the NR activity was lower than with NaCl in all cases, being their values between 30 and 86% of the activities reached in NaCl (Figure 4). Moreover, NR activity in *D. viridis* showed a significant decrease as the osmotic strength of the growth medium was increased.

pH of the reaction mixture

External pH strongly affected the NR activity, which was reduced to 50% in the range of pH tested in this work (6–9.5) (Figure 5). The maximal activity was reached at pH 8.5, which is the highest value of pH reported in the literarure for the *in situ* method. A pH range of the assay mixture from 8 to 9 appeared to be adequate for the estimation of the NR activity in *D. viridis*.

Reducing power

NADH is the main physiological source of reducing power for nitrate reduction in Chlorophyceae (Syrett, 1981), although nitrate reduction in other algae is NADPH-dependent. NADH in darkness can be obtained after glucose respiration with a potential yield of 12 NADH from each molecule of glucose. We tested the effect of increasing external reducing power in the form of NADH and glucose (Figure 6); *D. viridis* could not use glucose as a suitable source of reducing power under the assay conditions, since addition of glucose had no effect on the NR activity, but the addition of NADH highly increased the activity of the enzyme. Nevertheless, when NADH was present at 4.8 mM a very significant decay of the activity was detected.

NR activity vs NO_3^- incorporation

A comparison between NR activity measurement using the optimized medium and real NO_3^- consumption by *D. viridis* was carried out (Figure 7). Uptake of NO_3^- by exponentially growing *D. viridis* was linear during the 8 h of experiments ($r^2 = 0.96$, $p<0.01$). NR activity was estimated at 0, 4 and 8 h. There was no significant change in NR activity during this experiment (ANOVA, $p<0.1$). Mean NR value recalculated for 1 mM of

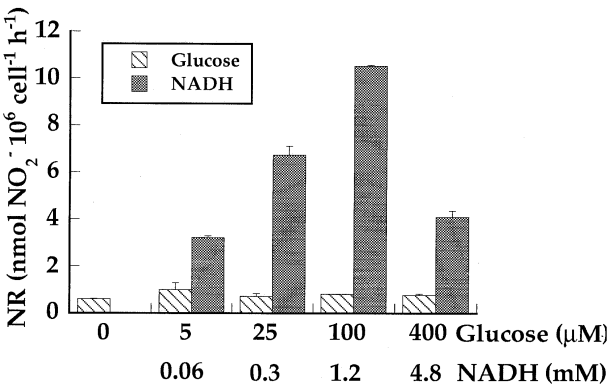

Figure 6. Effect of the addition of glucose and NADH as external reducing power sources on the NR activity of *D. viridis*. Glucose concentration was twelve-fold the NADH concentration, assuming a potential yield of the glucose of 12 NADH.

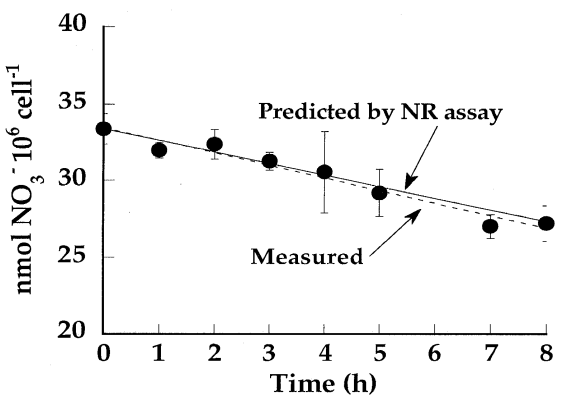

Figure 7. Comparison between the NO_3^- assimilation rate predicted by optimized NR assay (continuous line) and NO_3^- uptake measured as external NO_3^- decrease (linear fit as dashed line, $p<0.01$).

NO_3^- (the external concentration in the experiment) was 0.754 nmol NO_2^- 10^6 cell^{-1} h^{-1} and the slope of the linear curve fitted for NO_3^- disappearance was 0.817 nmol NO_3^- 10^6 cell^{-1} h^{-1}, both values being not statistically different (test for comparison of slopes, $p<0.01$).

Discussion

Permeabilisers and incubation time

The time course of nitrite production (Figure 1) shows a linear trend during the first 20 min; afterwards the conditions developed into the test tubes stopped the production, remaining constant the concentration of nitrite in the medium. Both NO_3^- and NADH are in excess concentration in the mixture, so their possible limitation is not responsible for the non-linearity of the assay after 20 min. Similar results have been reported for *Ulva rigida* (Corzo & Niell, 1991) with a linear phase during the first 30 min. The inactivation or degradation of the enzyme could explain the stop of the nitrite production after that period. Hochman et al. (1986) found a delay of 20 min before reaching a clear linear nitrite production rate using toluene-treated *Chlorella* cells. This delay could be due to the diffusion restrictions exerted by the viscosity of toluene.

Permeabilisers classically used in the *in situ* NR assays seem to operate by increasing membrane permeability, therefore favouring both the entry of nitrate to the reduction site and the exit of nitrite to the medium (Mann et al., 1979; Lawrence & Herrick, 1982). Among the alcohols, 1-propanol is the most effective in a number of species (Sym, 1984; Brinkhuis et al., 1989; Hernandez et al., 1993). Toluene is another of the permeabilising agents extensively used, giving better results than propanol in some cases (Hochman et al., 1986). Freezing and thawing has been used for the same purpose as a mechanical alternative to the utilization of organic compounds (Rhodes & Stewart, 1974; Mauriño et al., 1985). Estimated NR activity in *D. viridis* is higher when 1-propanol is used as permeabiliser agent, with an optimum concentration of 5% (v/v). This concentration is in the same range of previous reports, both for the marine macroalgae *Laminaria japonica* (Brinkhuis et al., 1989) and the freshwater microalgae *Monoraphidium braunii* (Corzo et al., 1991); nevertheless, the optimum concentration for the marine macrophytes *Ulva rigida* (Corzo & Niell, 1991) and *Porphyra umbilicalis* (Hernandez et al., 1993) were obtained using much lower 1-propanol concentrations (0.1%). Toluene severely reduces the production of nitrite at concentrations higher than 1% (v/v), probably due to its high viscosity that could prevent proper molecular diffusion.

KNO_3 supply

The estimated half-saturation constant (K_s) for NO_3^- in *D. viridis* was 5 mM. This value is much higher than those reported for several microalgae (Berges & Harrison, 1995b) and the brown macroalga *Laminaria digitata* (Davison & Stewart, 1984) using the *in vitro* assay, all of them in the μM range. Nevertheless, in *Ulva rigida*, a K_s in the mM range, using the *in situ* method, has also been reported (Corzo & Niell, 1991).

This suggests that the kinetic parameters are dependent of the method used for NR activity estimation (Hurd et al., 1995) and they are not usefull as K^m values like values from *in vitro* studies are. Higher K_s values in the *in situ* assay could be the result of more difficult accessibility for NO_3^- to the reduction site. It is interesting to point out that this high value for K_s in *D. viridis* is in the same range of the optimum concentration of NO_3^- for growth of halotolerant microalgae.

pH

The optimal pH for *in situ* NR estimation in *D. viridis* was found to be the highest value reported in the literature. In higher plants, these values ranged between 7.0 and 7.5 (Sym, 1984; Mauriño et al., 1986), for some microalgae, between 7.5 and 7.6 (Hochman et al., 1986), and for *Ulva rigida* the optimum was found at pH 8.0 (Corzo & Niell, 1991), close to the pH of seawater (8.2). Optimum pH for NR assay in *D. viridis* is 8.5–9.0, in the same range than optimum pH for growth of halotolerant species of *Dunaliella* (Borowitzka & Borowitzka, 1988).

Salinity

D. viridis is a halotolerant microalga with an optimal growth at 1 M NaCl, being able to grow over 4 M NaCl (Jiménez & Niell, 1991); nevertheless, Na^+ cell concentration remains low whatever is the external salinity of the medium (Katz & Avron, 1985). Due to the use of permeabilising agents in the *in situ* assay, it could be possible that those high concentrations of Na^+ and Cl^- may affect the activity of the enzyme; therefore, we tested three salinities (1, 2 and 3 M NaCl) and the substitution of the salt in the assays by an osmotic and non-ionic compound, glycerol, which is the natural 'osmoticum' in *Dunaliella*. Nevertheless, the use of glycerol in the NR assay was unsuccessful (Figure 4), reaching values between 30 and 86% of those obtained in NaCl. The reason for this decrease in the estimated NR activity could also be found in the high viscosity of glycerol. The lower activity found with increasing NaCl concentration may not be an effect of high NaCl levels in the reaction mixture, but a natural decrease in nitrogen assimilation ability according to the decrease in growth rate at high salinity (Jiménez & Niell, 1991).

Reducing power

NADH has been widely used as an external source of reducing power in NR assays both *in vitro* and *in situ*; but the use of NADH has some problems because it interferes with the full development of color in the diazotization of nitrite with sulfanilamide and N-(1-naphtyl) ethylendiamine dihydrochloride; therefore, several post-assay treatments have been developed (e.g. Scholl et al., 1974; Scheidler & Ninemann, 1986). In this work, active charcoal (Stulen, 1970) was used for removing NADH from the assay medium, and none of the problems mentioned by Scholl et al. (1974) were detected.

Corzo & Niell (1991) found an increase of 69% in the NR activity in *Ulva rigida* using glucose with respect to a control without external supply of reducing power. Glucose was not effective as a source of reducing power in the *in situ* NR assay in *D. viridis*.

It has also been found that excess NADH leads to an inhibition of nitrite production. The possibility of an inefficient removal of all the NADH by active charcoal and the subsequent inhibition of color development was discarded as we found that the post-assay charcoal treatment was able to completely remove the absorbance in the 340 nm region (NADH absorbing region). It was previously reported that NADH chemically inactivates the NR enzyme under *in vitro* conditions, both when NO_3^- was not present (Moreno et al., 1972; Aryan et al., 1983) and in the presence of NO_3^- (Berges & Harrison, 1995a). In our case, high levels of NADH in the assay medium led to a significant decrease of the NR activity. It is well established that NR in *Chlorella* is inactivated *in vivo* by NADH and cyanide (Lorimer et al., 1974; Pistorious et al., 1976). The significance of this effect acting *in vivo* under active photosynthesis is uncertain, although a control role in C-N interacting metabolism cannot be discarded. Maximal activity was found at 1.2 mM NADH being in agreement with the optimal range reported for the brown macroalgae *Fucus gardneri* by Hurd et al., (1995) using an improved *in vitro* assay.

As concluding remarks, the *in situ* method gives good reproducibility for its use in hypersaline systems since NaCl in the assay medium seems not to affect the activity of the enzyme. Moreover, the activity of the NR in *D. viridis* is dependent on the presence of NADH as source of reducing power, which can cause inhibition when is added in excess. Glucose is not an effective supply to generate reducing power in the species used. Finally, if optimal conditions for the assay are not

properly established, it will be imposible to predict nitrogen metabolism from NR estimations. In addition, if internal NO_3^- storage is being carried out, internal accumulation rate must be taken into account when the *in situ* method is being used as an estimation of nitrate assimilation.

Acknowledgements

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