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# AN *IN VITRO* NITRATE REDUCTASE ASSAY FOR MARINE MACROALGAE: OPTIMIZATION AND CHARACTERIZATION OF THE ENZYME FOR *FUCUS GARDNERI* (PHAEOPHYTA)[1]

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ABSTRACT

*Measurement of the activity of the enzyme nitrate reductase (NR) may provide a useful index of nitrogen metabolism in marine macroalgae. In several species, including* Fucus gardneri *P. C. Silva,* in vitro *assays previously failed to detect NR activity, necessitating the use of* in situ *(or so-called "*in vivo*") assays, which are more loosely controlled and lead to difficulties in assessing enzyme characteristics such as the half-saturation constant ($K_m$). In this paper, we describe an* in vitro *NR assay developed for* F. gardneri, *in which tissue was homogenized using liquid nitrogen prior to the assay. In contrast to previous studies, enzyme activity was always detectable in* F. gardneri *collected directly from the field at levels up to 30 nmol nitrate converted to nitrite·min$^{-1}$·g$^{-1}$ wet weight. The effect of a variety of compounds, commonly added to NR extraction buffers, were tested. Additions of protease inhibitors, bovine serum albumin, and ethylenediamine tetraacetic acid had no consistent effects on NR activity, while polyvinyl pyrrolidone, potassium ferricyanide, and flavin adenine dinucleotide significantly decreased activity. The half-saturation constant ($K_m$) for NADH was 0.18 (±0.05) mM and for nitrate, $K_m$ = 0.99 (±0.41) mM. Significant NR activity was detected without the addition of nitrate, suggesting that internal pools of nitrate averaging approximately 20 μmol $NO_3^-$·g$^{-1}$ wet weight were present in* F. gardneri *in February. The distribution of NR activity within the plant was highly variable between individuals, but activities were approximately 5-fold lower in the stipe than in midregions. In plants freshly sampled from the field, NR activity increased 7-fold from February to March, then fell to near-February levels by April. These changes in activity may correspond to seasonal changes in growth rate. The assay, optimized for* F. gardneri, *was used in several different macroalgal species from different taxa:* Porphyra *sp.,* Coralina vancouveriensis *Yendo,* Ulva *sp.,* Enteromorpha intestinalis *(Linnaeus) Nees,* Macrocystis integrifolia *Bory; and* Costaria costatum *(C. Agardh) Saunders. For all species tested, NR activity was detectable and, except for one species (*Porphya *sp.), was equal to or greater than activities measured by other workers using* in vivo *or* in vitro *assays for plants under similar conditions.*

*Key index words: Chlorophyta; enzyme activity;* Fucus gardneri; in vitro *assay; Phaeophyta; nitrate reductase; nitrogen; Rhodophyta; seaweed*

Seaweeds play an essential role in primary production and inorganic nutrient cycling in estuarine and marine coastal ecosystems (Mann 1973, Hanisak 1983, Duggins et al. 1989, Lavery and McComb 1991). Nitrogen is thought to be the nutrient most frequently limiting macroalgal production (Hanisak 1983, Lobban and Harrison 1994), with nitrate being the most abundant form of inorganic nitrogen in most neritic marine systems (Ryther and Dunstan 1971). Despite their importance in nitrogen processes, however, little is known about macroalgal nitrogen metabolism when compared to the large body of higher plant and microalgal literature on this subject. This deficiency is due, in part, to a variety of methodological difficulties in culturing macroalgae and performing uptake and assimilation experiments (Harrison and Druehl 1982, Hanisak 1983).

The enzyme nitrate reductase (NR, EC 1.6.6.1) catalyzes the reduction of nitrate to nitrite and is thought to be the rate-limiting step in the process that includes nitrate uptake and its subsequent reduction to ammonium (Solomonson and Barber 1990). The regulation of the enzyme is complex, potentially involving synthesis and degradation cycles (Solomonson and Barber 1990), redox and allosteric modulation (e.g. Kaiser and Brendle-Behnisch 1991), and phosphorylation (Huber et al. 1992). Furthermore, extraction of the enzyme is troublesome due to factors such as loss of enzyme cofactors during extraction (Vennesland and Solomonson 1972), inhibition of activity by phenolic compounds (Thomas and Harrison 1988), and the presence of endogenous proteases (Berges and Harrison 1995a). Despite these considerations, when NR assays are properly optimized, it has been possible to demonstrate a strong, quantitative relationship between NR activity and nitrate incorporation in marine diatoms (Berges and Harrison 1995a) as well as qualitative relationships between NR activity and nitrate uptake and growth rate in marine macrophytes (Davison and Stewart 1983, Davison et al. 1984).

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Several methodological problems that have hindered progress in macroalgal NR metabolism can be identified. In particular, traditional *in vitro* assays have failed to detect NR activity for several species tested (Thomas and Harrison 1988, Corzo and Neill 1992). Most workers have therefore relied on the so-called *in vivo* assay (Table 1), here termed *in situ* assays to distinguish them from truly *in vivo* techniques such as nuclear magnetic resonance (c.f. Corzo and Neill 1992). In cases where both *in vitro* and *in situ* methods have proved possible, higher activities have often been found using the *in situ* method (Table 1). However, the drawbacks of this method are the requirement for specially controlled environments (e.g. anaerobiosis; see Lillo 1983, Brinkhuis et al. 1989, but note also Gao et al. 1992) and assumptions about the permeability of cells to both substrate and products (Hog et al. 1983, Thomas and Harrison 1988). In the *in situ* method, factors such as substrate concentrations and effective pH are not known. As a result, kinetic constants (i.e. $K_m$ and $V_{max}$ values) are difficult to determine using *in situ* techniques, making studies of enzyme characteristics difficult. For both *in situ* and *in vitro* techniques, many researchers found it necessary to enhance NR activity to measurable levels by raising assay temperatures far greater than those encountered by the seaweed in the field (Table 1). The potential drawback of this approach is that there is clear evidence that the enzyme may be affected unpredictably; assay temperature is clearly a major and nonlinear factor (Kristiansen 1983, Davison and Davison 1987).

The key issues for seaweed NR assays appear to be adequate homogenization of the tough, rubbery thalli found in many species (see Eppley 1978, Thomas and Harrison 1988) and preservation and stabilization of NR activity during extraction (see Gegenheimer 1990). Significant improvement in homogenization has been noted by using liquid nitrogen to freeze the thallus before grinding; in fact, this is a routine method for higher plant NR extraction (see Huber et al. 1992) and for seaweed molecular biology and biochemistry (e.g. Gomez-Pinchetti et al. 1992, Mayes et al. 1992). The liquid nitrogen method has also been used to extract other enzymes from macroalgae (Küppers and Weidner 1980), and liquid nitrogen has been used to preserve seaweed samples prior to an *in situ* NR assay (Hernandez et al. 1993). Recent improvements in NR extraction from phytoplankton have highlighted the importance of inhibition of cellular proteases and removal of phenolic compounds (Berges and Harrison 1995a). Modifications have included additions of anionic detergent (Triton X-100), polyvinyl pyrrolidone (PVP), and bovine serum albumin (BSA). Again, most of these modifications have not been rigorously tested on seaweeds.

In this paper, we describe a simple, *in vitro* assay for measuring NR activity in macroalgae. The assay was optimized for freshly collected *Fucus gardneri* P. C. Silva, a species for which previous NR assays proved inadequate (Thomas and Harrison 1988; Table 1). The optimized assay was used to characterize the macroalgal enzyme in terms of kinetic constants and stability. The distribution of NR activity along adult thalli of *F. gardneri* was also investigated. The assay was then applied, in a preliminary study, to representatives from the Rhodophyta (*Porphyra* sp. and *Coralina vancouveriensis* Yendo), Chlorophyta (*Ulva* sp. and *Enteromorpha intestinalis* (L.) Nees), and Phaeophyta (*Macrocystis integrifolia* Bory and *Costaria costatum* (C. Aghard) Saunders).

## MATERIALS AND METHODS

Whole plants of *Fucus gardneri* P. C. Silva were collected between January and April 1993 from a rocky landfill site at English Bay, Vancouver, British Columbia, Canada (48°16.5′N, 123°0.5′W). Plants were collected from the waterline to minimize desiccation and were kept in seawater in the dark at 4° C until assayed, no longer than 15 h after collection and frequently less than 2 h.

*Assay optimization and characterization.* Tissue pieces of 0.2 g blotted wet weight were cut from the midregion of the thallus using a razor blade and immediately ground to a fine powder under liquid nitrogen in a mortar and pestle. Ground tissue was then homogenized briefly in a Teflon-glass homogenizer with 3.0 mL of ice-cold 200 mM phosphate extraction buffer (pH 7.9, based on Eppley 1978) in an ice-water slurry. All chemicals used in extractions and assays were obtained from Sigma Chemical Co. (St. Louis, Missouri). Initially, the phosphate extraction buffer contained 3% w/v BSA, which may act as a protease substrate and bind phenolic compounds that could inhibit NR activity (Ingemarsson 1987); 5 mM ethylenediamine tetraacetic acid (EDTA); 0.3% v/v PVP, which also adsorbs phenolics (Loomis and Bataille 1966); 0.3% v/v dithiothreitol, which prevents oxidation of thiol groups critical for enzyme activity (Cleland 1964); and 0.1% v/v Triton X-100, a nonionic detergent that disrupts cellular membranes to aid NR extraction (Tiller et al. 1984). Homogenates were kept on ice and assayed within 15–30 min. NR activity was determined by measuring the production of nitrite at a seawater temperature similar to that from which *Fucus gardneri* was collected (12° C) for 15–30 min. Homogenates were not centrifuged prior to the assay; a loss of 5–10% NR activity was observed when homogenates were centrifuged for 5 min at 750 × *g*. This loss would be acceptable for enzyme characterization, but for this study we were also interested in total NR activity. Assays were conducted in 5-mL plastic culture tubes in a total volume of 1 mL, using 200 μL of homogenate. Final concentrations of NADH and $KNO_3$ were 2 and 10 mM, respectively. Reactions were started with the addition of nitrate and stopped by placing tubes in boiling water and adding 1 mL 550 mM zinc acetate and 20 μL 825 mM phenazine methylsulphate to oxidize residual NADH, which could interfere with subsequent steps in the nitrite assay (Scholl et al. 1974). To correct for any internal nitrite present in the seaweed, a replicate reaction, stopped prior to the addition of nitrate, was conducted. Homogenates were centrifuged at 600 × *g* for 5 min. The supernatant was decanted, and the nitrite concentration was measured spectrophotometrically (Parsons et al. 1984). NR activity was expressed as $U \cdot g^{-1}$ wet weight, where 1 U = 1 μmol $NO_3$ reduced·$min^{-1}$.

To determine the effects of different components of the extraction buffer on NR activity, extracts were prepared without either EDTA, PVP, or BSA. The addition of a broad-spectrum protease inhibitor mixture was also tested (Gegenheimer 1990); final concentrations were 1.0 mM phenyl methyl sulfonyl fluoride, 1 mM benzamide, 1 mM benzamidine, 10 mM ethylene

glycol bis ($\beta$-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (or EGTA), and 1 $\mu g \cdot mL^{-1}$ each of leupeptin, antipain, and pepstatin A.

To verify that NR activity increased linearly with increasing amounts of seaweed homogenate, tissue samples from four plants were homogenized and the assay was conducted with 40, 80, 100, 200, and 400 $\mu$L homogenate.

Experiments to characterize the *Fucus gardneri* NR used the optimal extraction buffer, including EDTA, but not PVP or BSA. The use of NADPH as an electron donor by the *F. gardneri* NR was tested by substituting 2 mM NADPH for NADH in the reaction. The effect of flavin adenine dinucleotide (FAD) and potassium ferricyanide (FeCN) on NR activity was also tested by adding a final concentration of either 0.2 mM FeCN or 0.10 mM FAD to the reaction solution.

The stability of the extracted enzyme was examined by preparing four replicate homogenates from different plants and maintaining them on ice for 0, 0.25, 0.5, 1, and 2 h before assaying for NR activity.

NR activity was measured at concentrations of $KNO_3$ ranging from 0 to 20 mM and 0 to 5 mM NADH. Half-saturation constants ($K_m$) were calculated from nonlinear regressions using the curve-fitting package of SigmaPlot 5.0 (Jandel Scientific Co., San Rafael, California; see Berges et al. 1994).

*NR distribution.* Four whole, reproductive, plants of similar-sized *Fucus gardneri* (20 g wet wt), each with four dichotomies, were collected on 6 April 1993. For each plant, one dichotomy (ca. 8 cm long) was selected, and 0.2 g wet weight tissue samples were taken from each of the following distances from the basal region: base (stipe), 2 cm (mid a), 5 cm (mid b), and 8 cm (apices). NR activity of each sample was analyzed using the optimized assay.

*Species survey and NR induction.* In June 1993, a survey of NR activity in six species of intertidal algae was conducted using the method optimized for *Fucus gardneri.* Whole, hydrated plants of *Ulva* sp., *Enteromorpha intestinalis* (L.) Nees, *Porphyra* sp., *Coralina vancouveriensis* Yendo, *F. gardneri,* and *Costaria costatum* (C. Aghard) Saunders were collected from the intertidal region of Brady's Beach, Bamfield (48°50'N, 125°9'W) at 0845. Blades of *Macrocystis integrifolia* Bory were collected from Bamfield Inlet at 0910 by boat, and all plants were returned to the laboratory at Bamfield Marine Station within 1 h. Plants were maintained in flowing seawater with an irradiance of 1.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for up to 1.5 h. Homogenates were prepared within 2 h of collection and maintained on ice until the NR assay was conducted at an incubation temperature of 14° C.

Because many authors have performed assays after incubation of plants at high nitrate concentrations (Table 1), the effects of short-term exposure to increased nitrate concentration on NR activity was investigated in *Fucus gardneri.* In June 1993, three whole plants (ca. 10–15 g wet wt) were placed in a 4-L Pyrex beaker containing 4 L filtered (0.5 $\mu$m) seawater, with nitrate added to provide an initial concentration of 30 $\mu$M. Beakers were placed in a cold room set at 12° C with a constant irradiance of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 17 h, overnight. The nitrate concentration following incubation was 18 $\mu$M. NR activities were measured before and after incubation using the optimized assay.

All statistical comparisons were made using one-way ANOVA, testing for significance at $P < 0.05$ unless otherwise stated.

## RESULTS

Nitrite production was linear for up to 1 h, and NR activity increased linearly with increasing homogenate addition (data not shown). Neither the addition of protease inhibitors or BSA, nor the removal of EDTA from the extraction buffer, had a significant effect on NR activity ($P > 0.5$ in both cases, data not shown). The removal of PVP from the extraction buffer resulted in an activity that was 1.6 times higher than that of the control buffer ($P < 0.05$, data not shown).

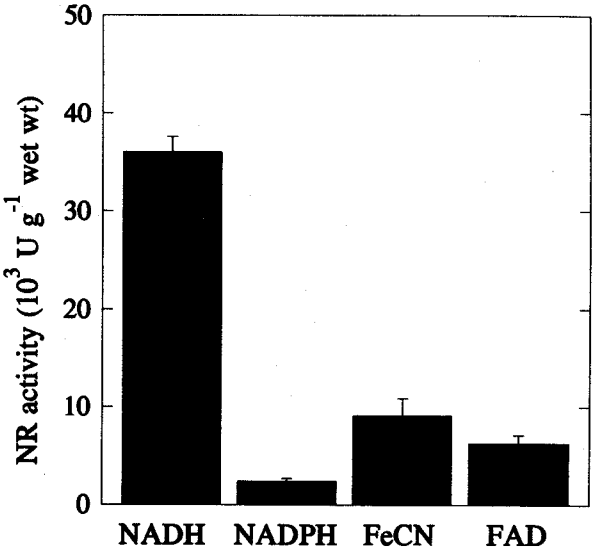


FIG. 1. Effects of different NR assay components on NR activity in *Fucus gardneri* extracts. NADH = control NR activity with 2 mM NADH as electron receptor, NADPH = activity with 2 mM NADPH in place of NADH, FeCN = activity with 0.2 mM potassium ferricyanide, and FAD = activity with 0.10 mM flavin adenine dinucleotide. Error bars represent ±1 SE of mean values (n = 3 separate homogenates).

When NADPH was used as the electron source instead of NADH, there was an 18-fold decrease in NR activity (Fig. 1, $P < 0.001$). The NR activities obtained when FeCN or FAD were added to the assay were 4 and 6 times lower than the control, respectively (Fig. 1, $P < 0.001$ in both cases). NR activity in homogenates held on ice showed no significant change over a 2-h period; the slope of a regression analysis was not significantly different from zero ($P > 0.2$, data not shown).

The apparent half-saturation constant ($K_m$) was 0.99 (± 0.41) mM for $KNO_3$ (Fig. 2A). No change in NR activity was seen for an addition of $KNO_3$ less than 0.02 mM, indicating substantial endogenous nitrate (inset Fig. 2A). To accurately estimate $K_m$ in the presence of this background, we used the method of Leatherbarrow (1990) to estimate $K_m$ and the concentration of endogenous nitrate. A background of 0.41 mM nitrate in the assay was calculated, corresponding to an *in vivo* nitrate concentration of approximately 20 $\mu mol\ NO_3 \cdot g^{-1}$ wet weight. If the 20 mM point was not included in the fit, a $K_m$ of 0.63 (±0.29) mM was calculated (dashed lines, Fig. 2A). For NADH, the half-saturation constant was 0.18 (±0.05) mM (Fig. 2B). If the 4 mM point was not included in the fit, the calculated $K_m$ was 0.24 (±0.03) mM (Fig. 2B).

The NR activity recorded in the stipe of *Fucus*

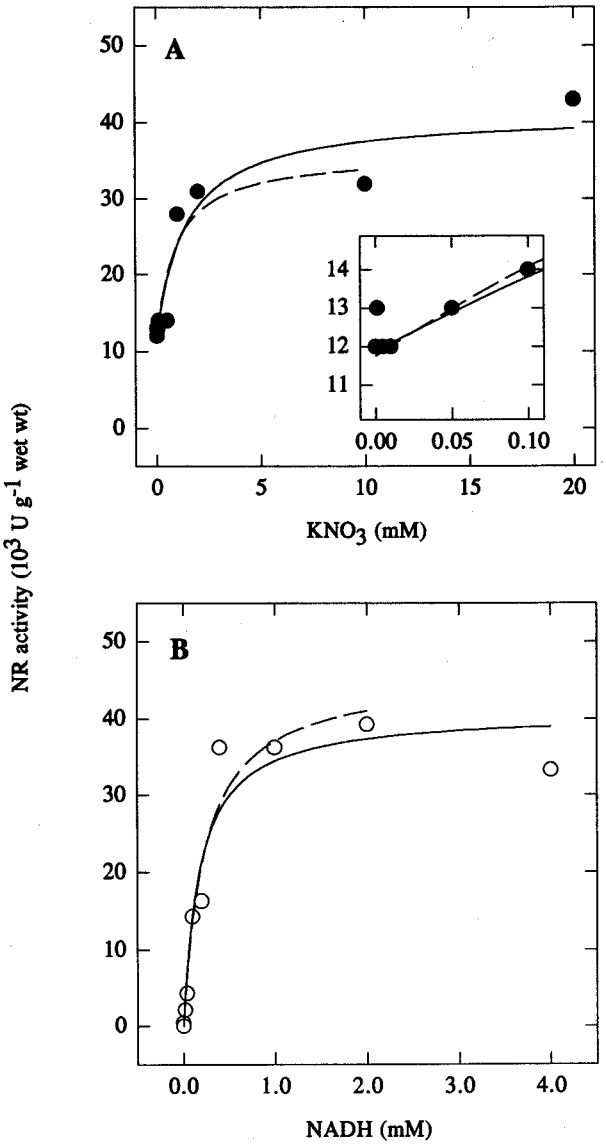


FIG. 2. Kinetic curves of NR activity in extracts from *Fucus gardneri* versus concentration of A) $KNO_3$ (with NADH concentration fixed at 2 mM) or B) NADH (with $KNO_3$ concentration fixed at 10 mM). Inset in A shows an enlargement of the concentration range from 0 to 0.10 mM $KNO_3$. Curves are fit to Michaelis-Menten models using nonlinear regression, as described in the text. Solid lines include all data points; dashed lines were fit removing the highest points (in A, 20 mM $KNO_3$, and in B, 4 mM NADH), as described in Results.

*gardneri* was significantly lower than that of the midregions ($P < 0.05$, Fig. 3). NR activity measured in the apical and midregions was not significantly different.

NR activities recorded for the midregions of *Fucus gardneri* using the optimized assay were plotted against time (Fig. 4). Activities increased significantly from the period between 10 February and 23 March ($P < 0.001$), with the activity on 23 March being 7 times greater than that in February. Between 23 March and 6 April, activity dropped to a value not significantly different from that measured on 10 February ($P > 0.5$).

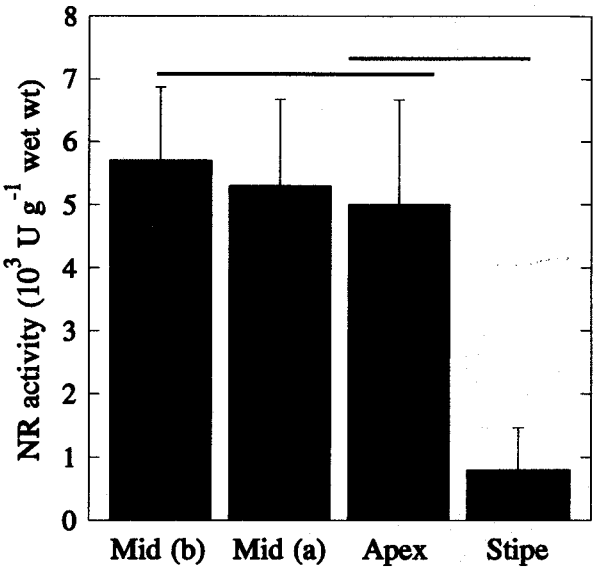


FIG. 3. Distribution of mean NR activity along thalli of *Fucus gardneri* measured in April 1993. Samples were taken at different distances along a single 8-cm-long dichotomy: base (stipe), 2 cm (mid a), 5 cm (mid b), and 8 cm (apex). Error bars represent ±1 SE of mean values (n = 4 separate plants). Lines above the bars join treatments that are not significantly different at $P > 0.05$.

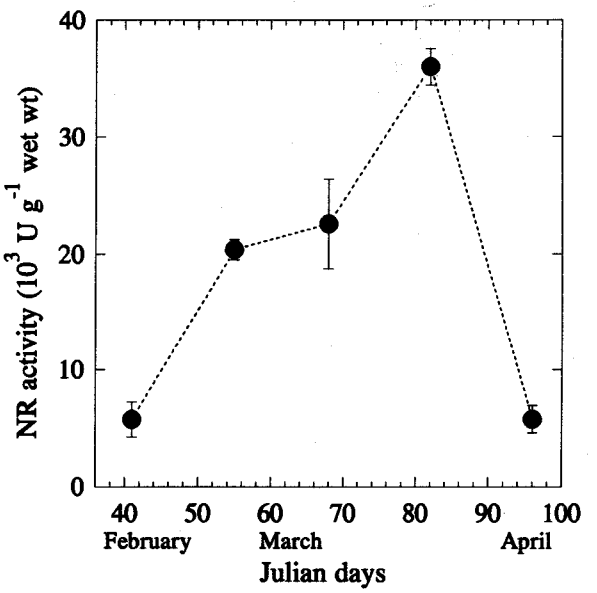


FIG. 4. Temporal variation in NR activity from the midregions of *Fucus gardneri* thalli between 10 February and 6 April 1993. Error bars represent ±1 SE of mean values (n = 4 separate plants).

TABLE 1. *Comparison of maximum nitrate reductase activities and assay conditions for seaweeds measured in the present (±1 SE) and previous studies. U = 1 μmol $NO_3$ reduced·min$^{-1}$. Seaweeds were freshly collected unless otherwise stated. * Activity has been converted from dry weight, assuming dw = 0.1 wet wt. NR activities estimated by Thomas and Harrison (1988) were converted to a wet wt basis using estimates of soluble protein from Thomas (1983):* Enteromorpha intestinalis, *10 mg protein·g$^{-1}$ wet wt;* Fucus gardneri, *12 mg protein·g$^{-1}$ wet wt; and* Porphyra perforata, *25 mg protein·g$^{-1}$ wet wt. This value (25 mg protein·g$^{-1}$ wet wt) was also used to convert values for* Porphyra yezoensis *given by Araki et al. (1979). ns = not stated, preinc. = preincubation. The* in situ *assay is often termed* in vivo *in the literature (see text for details).*

| Species | Assay | NR activity ($10^3$ U·g$^{-1}$ wet wt) | Assay temperature (° C) | Time of year | Other comments | Source |
|---|---|---|---|---|---|---|
| Chlorophyta | | | | | | |
| *Ulva lactuca* | *In situ* | 0.42* | 26 | Jan | Preinc. 10 mM $NO_3$ | Murthy et al. 1986 |
| *Ulva rigida* | *In situ* | 3.8 | 30 | ns | | Corzo and Niell 1991:fig. 1 |
| *Ulva fenestrata* | *In situ* | 5.9 | 16 | Summer | Preinc. 0.9 mM $NO_3$ | Gao et al. 1992:fig. 5 |
| | *In situ* | 29.3 | 16 | Winter | Preinc. 0.9 mM $NO_3$ | |
| *Ulva* sp. | *In vitro* | 9.3 (±1.6) | 14 | Jun | | Present study |
| *Enteromorpha intestinalis* | *In vitro* | 26.7 | Room temp. | Feb–Mar | | Thomas and Harrison 1988 |
| | *In situ* | 0.8 | Room temp. | Feb–Mar | | |
| | *In situ* | 4.2 | Room temp. | Feb–Mar | Preinc. 30 μM $NO_3$ | |
| *Enteromorpha intestinalis* | *In situ* | 13.8 | 22 | Aug | | Maier and Pregnall 1990:table 2 |
| Phaeophyta | | | | | | |
| *Giffordia mitchellae* | *In situ* | 10 | 20 | ns | Cultured | Weidner and Kiefer 1981 |
| *Macrocystis angustifolia* | *In vitro* | 3.3 | ns | Summer | | Haxen and Lewis 1981 |
| | *In vitro* | 11.7 | ns | Summer | Preinc. 1.8 mM $NO_3$ | |
| *Macrocystis integrifolia* | *In vitro* | 9.61 (±1.8) | 14 | June | | Present study |
| *Laminaria digitata* | *In vitro* | 3.3 | 30 | May–Jun | | Davison and Stewart 1984a |
| | *In vitro* | 5.3 | 10 | May–Jun | | |
| *Laminaria saccharina* | *In situ* | 4.8 | 10 | Aug–Dec | Provasoli ES enrichment | Davison and Davison 1987 |
| *Laminaria japonica* | *In situ* | 0.17 | 20 | Jun–Jul | | Brinkhuis et al. 1989 |
| *Laminaria japonica* | *In vitro* | 0 | 20 | Jun–Jul | | |
| *Costaria costatum* | *In vitro* | 10.7 (±1.9) | 14 | Jun | | Present study |
| *Sargassum filipendula* | *In situ* | 5.8 | 22 | Aug/Oct | | Maier and Pregnall 1990 |
| *Fucus gardneri* | *In vitro* | 0 | Room temp. | Feb–Mar | | Thomas and Harrison 1988 |
| | *In situ* | 0 | Room temp. | Feb–Mar | | |
| | *In situ* | 6.0 | Room temp. | Feb–Mar | Preinc. 30 μM $NO_3$ | |
| *Fucus gardneri* | *In vitro* | 5.73 (±1.51) | 12 | Feb | | Present study |
| | | 35.99 (±1.58) | 12 | Mar | | Present study |
| | | 1.5 (±0.4) | 14 | Jun | | Present study |
| Rhodophyta | | | | | | |
| *Petroglossum nicaeense* | *In situ* | 0.4 | 25 | ns | | Dipierro et al. 1977 |
| *Porphyra yezoensis* | *In vitro* | 33.2 | 25 | | | Araki et al. 1979: fig. 2 |
| *Porphyra perforata* | *In vitro* | 20.8 | Room temp. | Feb–Mar | | Thomas and Harrison 1988 |
| | *In situ* | 25.0 | Room temp. | Feb–Mar | | |
| | *In situ* | 41.7 | Room temp. | Feb–Mar | Preinc. 30 μM $NO_3$ | |
| *Porphyra umbicalis* | *In situ* | 19.2–158 | 10, 20 | Dec–Mar | | Hernandez et al. 1993 |
| *Porphyra* sp. | *In vitro* | 4.0 (±0.96) | 14 | Jun | | Present study |
| *Corallina vancouviensis* | *In vitro* | 22.6 (±1.31) | 14 | Jun | | Present study |

For each of six species tested in June 1993, significant NR activity was measured using the optimized assay for *Fucus gardneri* (Table 1). Activities ranged from $1.5 \times 10^{-3}$ μmol $NO_2 \cdot g^{-1}$ wet weight·min$^{-1}$ in *F. gardneri* to $22.6 \times 10^{-3}$ μmol $NO_2 \cdot g^{-1}$ wet weight·min$^{-1}$ for *Coralina vancouveriensis.* Coefficients of variation ranged from 6% (*C. vancouveriensis*) to 29% (*F. gardneri*).

For the brief exposures used, there was no significant difference in the NR activity of *Fucus gardneri* held overnight in 30 μM nitrate compared to freshly collected plants ($P > 0.5$, data not shown).

## DISCUSSION

*Assay optimization and characterization.* The present study shows clearly that with proper optimization *in vitro* assays for NR can be easily performed on field-collected *Fucus gardneri.* The induction period in nitrate-enriched seawater described by Thomas and Harrison (1988) was not required, and activity was

easily detected at the ambient seawater temperature. The method was also successful in detecting NR activity for five other species, from the three major seaweed groups. For all but one of these species (*Porphyra* sp.), NR activities were similar to, or greater than, those of other workers (Table 1), demonstrating the robustness and potential use of this technique. Activities recorded for species other than *F. gardneri* must be interpreted cautiously, however, because no attempt was made to optimize activity in these cases.

The main improvement of this method over previous *in vitro* NR methods was the use of liquid nitrogen to freeze the thallus. This step makes homogenization (grinding) rapid and simple (Küppers and Weidner 1980). If liquid nitrogen is not available, dry ice in ethanol was also useful, although not as reproducible (data not shown). Liquid nitrogen freezing stops all enzyme activity and lowers the risk of enzyme inactivation by proteases. Long-term preservation of samples (up to 2 weeks) has been demonstrated for marine phytoplankton (Berges and Harrison 1995a) and apparently works for macrophytes as well (Hernandez et al. 1993).

Using both *in vitro* and *in vivo* assays, Thomas and Harrison (1988) recorded no NR activity for field collected *Fucus gardneri* from the same site as used in this study and at the same time of year. These authors suggested that the relatively high seawater ammonium concentrations (4 $\mu$M) suppressed NR activity, despite seawater nitrate concentrations of 17 $\mu$M. Surface seawater concentrations of nitrate and ammonium at the time of our experiments were similar to those of Thomas and Harrison (1988) at ca. 6–10 and 1–3 $\mu$M, respectively (K. Yin, unpubl. data). In this study, NR activity was always recorded for *F. gardneri*, despite the relatively high concentrations of ammonium in seawater. The presence of ammonium does not appear to inhibit nitrate uptake by members of the Phaeophyta; simultaneous uptake of both ions has been previously noted (Haines and Wheeler 1978, Topinka 1978, Harrison et al. 1986, Thomas and Harrison 1987). Based on this observation, we suggest that the extraction technique used by Thomas and Harrison (1988) either degraded or inactivated the enzyme or released metabolites that interfered with the NR assay. Similarly, Corzo and Niell (1992) were unsuccessful in developing an *in vitro* method for *Ulva rigida*, whereas activity was easily detected for *Ulva* sp. in this study (Table 1).

NR activity showed distinct requirements and characteristics in *Fucus gardneri*. The beneficial effects of PVP found by Thomas and Harrison (1988) were not observed in this study; in fact, a significant decrease in NR activity was observed upon addition of PVP. It is possible that homogenization in liquid nitrogen helps prevent oxidation of phenolic compounds, thus minimizing their interference in the assay. FAD additions have variable effects, sometimes enhancing NR activity (Everest et al. 1984), but this was clearly not the case for *F. gardneri*. FeCN activates NR in some green algae (Pistorius et al. 1976), but this does not occur in marine diatoms (Serra et al. 1978, Berges and Harrison 1995a) or in *F. gardneri*.

In most higher plants and phytoplankton species, $K_m$ values for NADH are considerably lower than those of 0.18 mM recorded in this study and typically in the order of 10–20 $\mu$M (e.g. Eppley et al. 1969, Amy and Garrett 1974, Wray and Fido 1990). There are few examples of kinetic constants for NR determined in macrophytes, probably due to the difficulties inherent in specifying a concentration using *in situ* methods. However, using an *in vitro* method for induced NR activity, Thomas and Harrison (1988) recorded $K_m$ values in the range of 32–50 $\mu$M for three species (*Porphyra perforata, Fucus gardneri,* and *Enteromorpha intestinalis*), which are also lower than values obtained in this study. In many phytoplankton species, high NADH concentrations (>0.2 mM) can inhibit NR activity (Serra et al. 1978, Berges and Harrison 1995a), but this is apparently not the case for *Fucus gardneri*; although the 4-mM data point appears lower, kinetic constants do not change significantly if this point is dropped (Fig. 2B). As is typical in chromophytic algae, but not in green algae or higher plants, the enzyme appears to require NADH alone (Syrett 1981). The low activity found with NADPH may indicate the presence of another NR isozyme active with NADPH, the dephosphorylation of NADPH to NADH, or the conversion of NADPH to NADH by a transhydrogenase (see Jackson 1991).

The half-saturation constant of 0.99 mM is in the same range as that previously recorded for *Fucus gardneri, Enteromorpha intestinalis* (0.5 and 0.25 mM, respectively; Thomas and Harrison 1988) and *Porphyra yezoensis* (0.12 mM; Araki et al. 1979) but is an order of magnitude greater than that recorded by other workers (44 $\mu$M for *Laminaria digitata,* Davison and Stewart 1984a; 30 $\mu$M for *P. perforata,* Thomas and Harrison 1988). Typical higher plant and microalgal enzymes have $K_m$ values in the range of 10–200 $\mu$M (Serra et al. 1978, Packard 1979, Wray and Fido 1990). The reason for these order-of-magnitude variations among macroalgal $K_m$ values for $KNO_3$ is unknown, but an intriguing possibility is the existence of two or more separate NR isozymes with different $K_m$ values. This has recently been shown to be the case for a cyanobacterium that has a low-affinity NR with a $K_m$ of 50 $\mu$M and a high-affinity enzyme with a $K_m$ of 5–25 mM (Martin-Nieto et al. 1992). There are clearly differences between cyanobacteria and algal nitrogen metabolism, and that the $K_m$ values fall within the same two ranges may be pure coincidence. However, it is also possible that two or more NR isozymes exist but have not

been detected for macroalgae because the $KNO_3$ concentrations used were not sufficiently high (Davison and Stewart 1984a), or there was insufficient detail in the lower concentration ranges (Thomas and Harrison 1988, this study). The high NR activity recorded at 20 mM $NO_3$ indicates that substrate saturation of NR activity may not have been reached at the 10-mM concentration used in the assay (Fig. 2A). Interestingly, juvenile sporophylls of *Laminara saccharina* show biphasic NR activity at low $KNO_3$ concentrations (0–50 $\mu$M); $K_m$ = 1.9 $\mu$M for $KNO_3$ concentrations of 0–15 $\mu$M, and $K_m$ = 10.5 $\mu$M for concentrations of 15–50 $\mu$M (Wheeler and Weidner 1983). NR activity for *L. saccharina* juveniles did not reach a maximum rate at the highest $KNO_3$ concentration used (50 $\mu$M; Wheeler and Weidner 1983). This observation lends support to the existence of more than one NR enzyme in some seaweeds.

A diversity of substrate and extraction requirements for NR has been previously demonstrated for macroalgae (Thomas and Harrison 1988). Before conclusions about NR activity can be made, it is essential that optimal conditions are established for the test species and also verified under different treatment conditions. One factor not considered in this study was the effect of light on NR activity; NR shows daily variations in activity, with maximum rates occurring during the light period and minimal activity in the dark (Weidner and Kiefer 1981, Davison and Stewart 1984b, Gao et al. 1992). Because *Fucus gardneri* in this study was left in the dark for up to 15 h prior to the NR assay, it is possible that activities measured were underestimates. However, in recent work, NR activities measured for *F. gardneri* following 6 h incubation under an irradiance of 170 $\mu$mo·$m^{-2}$·$s^{-1}$ were not different from values reported in this paper (B. Kerin and C. Hurd, unpubl. data).

*NR distribution.* For *Fucus gardneri,* NR activity of the mid- and meristematic apical regions were similar, with lower activities being recorded in the basal region. Nitrate uptake rates of *Fucus* sp. were also high in the apical meristematic and the midregion but low in the stipe (Wallentinus 1984), which corresponds to the distribution of NR activity recorded for *F. gardneri* in this study. Davison and Stewart (1984b) similarly found low NR activities in the stipe region of the kelp *Laminaria digitata.* However, in contrast to *F. gardneri,* NR activities in the meristematic region of *L. digitata* were also low compared to mature tissue; for this species, nitrate is assimilated in the mature thallus and translocated as organic nitrogen to the basal meristem, which has a high nitrogen requirement for growth (Davison and Stewart 1983, 1984b). These results demonstrate that care must be taken when choosing regions of morphologically complex macroalgae for assay. Interplant variation in NR activity was high for all regions of *F. gardneri.* This variation is not surprising, however, as similar variation is commonly observed for nitrate uptake by morphologically complex macroalgae and may reflect adaptations to the rapidly fluctuating environment in which they naturally grow (Harrison et al. 1986).

*NR induction and seasonal variation in NR activity.* There was no significant increase in NR activity of *Fucus gardneri* following the 17-h incubation in nitrate-rich seawater, in contrast to the previous observations of Thomas and Harrison (1988), where NR activity in this species could not be detected without prior incubation. This may have been due to the relatively short incubation period used in this study, compared to the 1–3 days used by Thomas and Harrison (1988). For the red alga *Porphyra perforata,* a 2–3-day induction period in seawater containing high concentrations of nitrate (50 $\mu$M) was also required before increased NR activity was observed (Thomas and Harrison 1985). Further, our incubation experiment was conducted in June at a time when NR activity was low compared to February and March (Table 1); the low activity was probably due to the undetectable concentrations of nitrate in surface waters near Bamfield during the summer months (C. Hurd, unpubl. data; Smith et al. 1983). Low levels of NR activity were recorded for *Porphyra perforata* that was cultured in nitrate-free seawater (Thomas and Harrison 1985). Similarly, for *Laminaria digitata* low NR activities corresponded to low seawater nitrate concentrations and nitrogen-limited growth (Davison et al. 1984). Maintaining high levels of NR at times when nitrate is not available may be wasteful in energetic terms (Thomas and Harrison 1985).

For phytoplankton, NR activity has been demonstrated to be a useful indicator of nitrate incorporation (note that in microalgae, incorporation is a functional definition involving increases in filter-retained particulate nitrogen; see Berges and Harrison 1995a). Similarly, Davison et al. (1984) demonstrated that for the kelp, *Laminaria digitata,* seasonal changes in NR activity were correlated with variations in seasonal growth rate. For *Fucus gardneri,* the seasonal increase in growth rate occurred between February and March (Ang 1991). The increase in NR activity observed for *F. gardneri* over the period 10 February to 23 March agreed with the proposed late winter increase in metabolism. The reason for the decrease in NR activity on 6 April was not clear, however, as seawater nitrate concentrations (K. Yin, unpubl. data) and growth rates (Ang 1991) remained high at this time.

The usefulness of NR activity depends on the demonstration of relationships between enzyme activity and component processes of nitrate metabolism under controlled conditions (Berges and Harrison 1995a, b). This has not yet been accomplished for marine macrophytes, although correlations have been found (Davison et al. 1984). In theory, NR activity determined *in vitro* represents a maximal

activity; substrate availability or other regulatory mechanisms may cause NR activity to be lower than this *in vivo*. The determination of a true *in vivo* activity is problematic. Furthermore, in multicellular organisms, inter- and intracellular pools result from uptake into the intercellular spaces and transport into cells. This means that at different times components of nitrate uptake and not nitrate reduction may limit incorporation of nitrogen (see e.g. Imgemarsson 1987, Campbell 1988). In certain species of microalgae, the *in vitro* NR activity is quantitatively related to *in vivo* nitrate incorporation (Berges and Harrison 1995a, b), but the situation in macroalgae has yet to be rigorously examined.

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