

FIELD ASSAYS FOR MEASURING NITRATE REDUCTASE ACTIVITY IN *ENTEROMORPHA* SP. (CHLOROPHYCEAE), *ULVA* SP. (CHLOROPHYCEAE), AND *GELIDIUM* SP. (RHODOPHYCEAE)¹

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***In situ* and *in vitro* nitrate reductase (NR) activity assays designed for use in the field on *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. are described. In optimizing each assay, a variety of compounds and assay conditions were tested for their ability to extract NR and preserve its activity. *Enteromorpha* sp. had similar levels of *in vitro* NR activity after exposure to the *in situ* assay buffer, demonstrating that neither NR induction nor activation likely occurs during the *in situ* assay. Storing freshly collected *Enteromorpha* sp. led to a reduction in NR activity over time. However, the use of liquid nitrogen to freeze algal tissue on site and subsequent storage at -80°C preserved NR activity and allowed for later laboratory use of the optimized *in vitro* assay. Application of the *in situ* and *in vitro* assays to stands of *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. in the field consistently found NR activity. *In situ* NR activity over 9 consecutive days in January demonstrated that *Enteromorpha* sp. responds to increases in nitrate availability. The influence of light on diel patterns of *in vitro* NR activity in the field was demonstrated for the first time as well. For the three species tested, these two assays provide a reliable tool for field investigation of the interaction between environmental signals (e.g. nutrient levels) and physiological signals (e.g. tissue metabolite levels) on nitrate reduction.**

Key index words: *Enteromorpha* sp.; estuary; *Gelidium* sp.; nitrate reductase; macroalgae; Mobile Bay; *Ulva* sp.

Abbreviation: ASW, artificial seawater; FSW, filtered seawater; NR, nitrate reductase

Macroalgal production is frequently limited by the availability of inorganic nitrogen (Lapointe et al. 1992, Wheeler and Bjornsater 1992, Lobban and Harrison 1994, Pedersen and Borum 1996). When inorganic nitrogen is available, it is often in the form of ni-

trate (NO_3^-) (Chapman and Craige 1977, Probyn and McQuaid 1985, Fujita et al. 1989). Depending on the locale, the supply of NO_3^- can vary substantially over time and occur in pulses whose duration and intensity are unpredictable (Chapman and Craige 1977, Nixon and Pilson 1983, Fujita et al. 1989). In upwelling regions, the supply of NO_3^- is often linked to the intensity and duration of upwelling events (Fujita et al. 1989). In estuaries, changes in the amount of NO_3^- often reflect changes in river discharge (Wafar et al. 1989, Selmer and Rydberg 1993, Ogilvie et al. 1997).

Because inorganic nitrogen can limit macroalgal growth, the capacity to take up and assimilate NO_3^- when it becomes available can determine macroalgal growth rates. In turn, the capacity to take up and assimilate NO_3^- depends on how quickly NO_3^- can be removed from the water column and either stored as inorganic nitrogen or reduced and assimilated. Particularly for ephemeral macroalgae (e.g. *Enteromorpha* sp., *Ulva* sp.), which can store only limited amounts of inorganic nitrogen (Rosenberg and Ramus 1982, Duke et al. 1987), the ability to use a pulsed supply of NO_3^- for growth will depend on the rate of NO_3^- reduction and assimilation.

Nitrate must be reduced to ammonium (NH_4^+) before it can be assimilated into amino acids. This reduction is a two-step process. Nitrate reductase (NR) is the enzyme responsible for catalyzing the initial reduction of NO_3^- to nitrite (NO_2^-), whereas nitrite reductase catalyzes the reduction of NO_2^- to NH_4^+ (Solomonson and Barber 1990). NR fits many of the criteria for a rate-limiting enzyme, and the reduction of NO_3^- to NO_2^- is thought to be the step that limits the rate of NO_3^- assimilation (Solomonson and Barber 1990, Berges et al. 1995, Berges 1997). Consequently, for macroalgae to fully use a pulsed supply of NO_3^- , levels of NR must be either maintained in the absence of NO_3^- or quickly elevated when NO_3^- becomes available.

Although much work has been done on the factors that regulate NR activity in higher plants and microalgae (Berges 1997, Campbell 1999), considerably less work has focused on macroalgae (Davison and Stew-

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art 1984a,b, Gao et al. 1992, Lopes et al. 1997), and none of this work was conducted in the field. Elucidating the macroalgal enzymatic response to a pulsed supply of NO_3^- is needed to develop a clearer understanding of nitrogen limitation in coastal waters and to reliably predict macroalgal rates of NO_3^- assimilation. In addition, species-specific differences in the enzymatic response to the availability of NO_3^- may help to explain why some species are more prolific in waters where the supply of NO_3^- is variable.

To investigate how environmental signals in the water column interact with physiological signals from metabolic pathways to determine levels of NR activity, a method for measuring NR activity in the field is needed. A few assays for measuring NR activity in macroalgae have been developed (Dipierro et al. 1977, Corzo and Neill 1991, Hurd et al. 1995). However, these assays were optimized for use in the laboratory, and their use in the field has not been demonstrated. For an assay to record the NR activity of macroalgae in the field, the assay must be either conducted on site or in the laboratory using tissue collected and transported in a manner that preserves NR activity.

NR activity can be measured as the amount of NO_2^- produced over time using either an *in situ* or an *in vitro* assay. The main difference between the two approaches is the preparation of the tissue. During an *in situ* assay, whole algal tissue is permeabilized with a detergent (e.g. Triton X-100) or an alcohol (e.g. *n*-propanol), allowing a buffered assay solution containing NO_3^- to seep into intact algal cells where, using endogenous NADH, the NO_3^- is reduced by NR. Because further reduction of NO_2^- requires reduced ferredoxin, a product of photosynthesis, *in situ* assays are performed in the dark. The need for anaerobic conditions during *in situ* assays has been proposed as well (Corzo and Neill 1991) but never tested in macroalgae. The major drawback to the *in situ* assay is that NO_2^- release may be limited not only by NR activity, but also by the diffusion rates of NO_3^- into the cells and NO_2^- out of the cells (Thomas and Harrison 1988, Hurd et al. 1995). In an *in vitro* assay, the algal tissue is homogenized to liberate NR from the tissue and suspend the enzyme in a buffered extraction solution. It is the preservation and stabilization of NR at this step that has presented one of the biggest challenges to using *in vitro* assays on macroalgae (Hurd et al. 1995). The activity of the NR preserved in the extraction step is then measured during the assay step. In the assay step, an aliquot of extract is incubated in a buffered assay solution containing NO_3^- and a source of reducing power (NADH), and the NO_2^- produced is quantified.

Here we demonstrate that storing freshly collected algal tissue reduces NR activity. We then present a simple *in situ* NR assay optimized for use in the field on *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. and demonstrate that neither NR induction nor activation likely occurs in *Enteromorpha* sp. during an *in situ* assay. We also present an optimized *in vitro* NR assay for

use with *Enteromorpha* sp. and *Gelidium* sp. and demonstrate that liquid nitrogen can be used to freeze *Enteromorpha* sp. tissue on site for later analysis in the laboratory without a loss in activity. We then apply the *in situ* and *in vitro* assays to *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. stands in the field. Finally, we use the *in situ* assay to track NR activity over 9 days and the *in vitro* assay to track NR activity over 24 h in field stands of *Enteromorpha* sp. and demonstrate that *Enteromorpha* sp. is highly responsive to fluctuations in nitrate and light availability.

MATERIALS AND METHODS

Collection. *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. were collected from below the waterline off the rock jetties and seawalls surrounding the east end of Dauphin Island (AL, USA) at the mouth of Mobile Bay (30°15.015' N, 88°04.759' W) from January until May in 2000 and 2001. Collected thalli were either transported to the Dauphin Island Sea Lab in water from the collection site or quickly rinsed with deionized water and NR activity assayed in the field. Thalli transported to the laboratory, a process that took less than 10 min, were rinsed upon arrival. Rinsed thalli were blotted dry and either immediately assayed for NR activity or frozen in liquid nitrogen and stored at -20°C or -80°C for later analysis. When evaluating the effect of short-term storage on fresh tissue, the thalli were held as described below.

Evaluation of short-term storage of fresh tissue on NR activity. To assess the effect of short-term storage of fresh tissue on NR activity, macroalgal tissue was collected, divided into replicate groups (five per storage treatment), and *in situ* NR activity assayed over time. The stored tissue was held at 10°C in the dark either in seawater (SW) collected at the site or between paper towels moistened with SW. *In situ* assays then were performed on the stored tissue after 0, 1, and 2.5 h.

In situ assay optimization. The optimized *in situ* assay buffer contained 2.25% (*Enteromorpha* sp. and *Ulva* sp.) or 3% (*Gelidium* sp.) (v/v) *n*-propanol and 30 mM potassium nitrate (KNO_3) in 20 practical salinity units (psu) artificial seawater (ASW; Marine Enterprises International, Inc., Baltimore, MD, USA) at pH 8.2. The assay was based on the *in situ* assay developed by Dipierro et al. (1977). Assays were performed with 0.10–0.15 g fresh weight (FW) of algal tissue in 5 mL of assay buffer. In the laboratory, assays were conducted in a dark 30°C water bath for 1 h unless indicated otherwise. After 1 h, the assay tubes were placed in a $>80^\circ\text{C}$ water bath for at least 5 min to denature algal proteins and end the assay. Tubes placed in a $>80^\circ\text{C}$ water bath at time 0 served as controls for NO_2^- already present in the algal tissue. At least three replicates were run for each treatment along with an equal number of controls.

After the samples had cooled to room temperature, NO_2^- concentration was determined colorimetrically (Parsons et al. 1984). A 0.5-mL aliquot was removed from each sample, and 0.5 mL of 1% (w/v) sulfanilamide in 1.2 N HCl was added. After 2 min, 0.5 mL of 0.01% (w/v) *n*-(1-naphthyl)-ethylenediamine (NEDA) was added. Finally, particulate matter was removed by centrifugation (13,600g) for 10 min at room temperature, and absorbance was measured at 543 nm against a standard NO_2^- curve. The amount of NO_2^- found in the controls was subtracted from the amount of NO_2^- measured in the samples and enzyme activity expressed as $\text{U}\cdot\text{g}^{-1}$ FW ($\text{U} = 1 \mu\text{mol } \text{NO}_3^- \text{ reduced}\cdot\text{min}^{-1}$).

To reduce the time between collection and the start of the assay, tissue mass was measured after the assay reaction had been stopped. FWs were determined after tissue samples had been pressed firmly between paper towels. Dry weights were measured after 24 h at 80°C . After 24 h at 80°C , dry weights averaged 12.0% of FW for *Enteromorpha* sp. (95% confidence interval (C.I.) = 2.7%, $n = 113$), 23.1% of FW for *Ulva* sp. (95% C.I. = 0.8%, $n = 39$), and 25.5% of FW for *Gelidium* sp. (95% C.I. = 2.3%, $n = 27$).

A range of buffers, salinities, pHs, *n*-propanol concentrations, and KNO_3 concentrations were tested to identify which assay conditions produced the highest NR activity. The capacity of exogenous NADH to increase NR activity was also tested. The substitution of DMSO for *n*-propanol and the need for anaerobic conditions during the assay were separately tested as well. Anaerobic conditions were generated by degassing the assay buffer using a vacuum pump and performing the assay in capped crimp-top vials sparged for 10 min with nitrogen before and after the addition of algal tissue. When measuring the production of NO_2^- over time, assays were performed at a starting volume of 7 mL with 0.5-mL aliquots removed at 0, 20, 40, 60, 90, 120, 150, and 180 min. To test the effect of temperature on NR activity, the assay was performed at 13.7, 14.5, 19.2, 23.3, 29, 32.5, and 39.8° C.

When the *in situ* assay was performed in the field, sample tubes were held at ambient water temperature in a flooded Pelican 1200 case (Pelican Products, Torrance, CA, USA). The Pelican case was weighted and submerged at the site for 1 h. In the field, the addition of 50 μL of 1.2 N HCl per 0.5 mL of assay buffer was tested as an alternative to immersion in $>80^\circ\text{C}$ water as a means of ending the assay.

Nitrite uptake. The possibility that macroalgal tissue might take up NO_2^- from the assay medium during an *in situ* assay was tested. Nitrite uptake experiments were performed under the conditions described above for the optimized *in situ* assay with the exception that NO_2^- replaced NO_3^- in the assay buffer and the temperature was lowered to 25° C (ambient water temperature). A range of NO_2^- concentrations (0, 5, 10, 50, and 200 μM) were tested with and without 2.25% *n*-propanol added to the assay buffer. Tubes containing no algal tissue served as controls.

In vitro assay optimization. The *in vitro* assay presented here is modified from a higher plant assay developed by Duke and Duke (1978) and a macroalgal assay developed by Hurd et al. (1995). In the optimized *in vitro* assay, algal tissue (0.20–0.25 g FW) was ground into a powder in liquid nitrogen using a mortar and pestle. The algal powder was then suspended in chilled extraction buffer (1 mL of extraction buffer per 0.1 g of algal tissue). The final extraction buffer contained 2% (w/v) casein, 1 mM cysteine, 5 mM KNO_3 , 1 μM FAD, and 5 mM EDTA in 100 mM phosphate buffer adjusted to pH 7.8.

Extracts were held on ice for ~ 10 min before the assay step. The optimized *in vitro* assay buffer contained 20 mM phosphate buffer (pH 8.0), 10 mM KNO_3 , and 0.2 mM NADH. Aliquots of assay buffer (1.8 mL) were warmed in a 30° C water bath and the assay started with the addition of extract (0.2 mL). Assays were run for 30 min and stopped by immersion in a $>80^\circ\text{C}$ water bath. Assay tubes that were immediately heated to $>80^\circ\text{C}$ served as the controls for background levels of NO_2^- . Each extraction was assayed in triplicate with the average of the three minus the control serving as one replicate.

Tubes were cooled to room temperature, and phenazine methosulfate was added to a final concentration of 25 μM to oxidize any residual NADH, which can inhibit the colorimetric reaction for NO_2^- (Scholl et al. 1974). After 20 min, 0.5 mL of 1% (w/v) sulfanilamide in 1.2 N HCl was added followed 2 min later by 0.5 mL of 0.01% (w/v) NEDA. The samples were then centrifuged at 13,600g for 10 min at room temperature to pellet any algal pieces and the casein, which precipitates with the addition of the acidic sulfanilamide solution. Finally, the absorbance of each sample was measured at 543 nm.

Several different extraction techniques and extraction buffer recipes were tested to identify the combination that would produce the highest NR activity. *In vitro* NR activity after extraction in 2% BSA, 2% casein, and no additional protein was compared. In addition, 100 mM Tris was tested as a substitute for 100 mM phosphate buffer in the extraction buffer. Homogenizing the tissue with an Omni 2000 homogenizer (Omni International, Warrenton, VA, USA) was tested as an alternative to grinding in liquid nitrogen. The ability of 0.1% Triton X-100 and 0.3% polyvinyl pyrrolidone (PVP) to enhance extraction of NR was also tested. Finally, centrifuging (13,600g) the extract

for 1 min at room temperature to clarify the extract before introduction into the *in vitro* assay buffer was tested.

In optimizing the *in vitro* assay buffer, a range of NADH concentrations and a range of pHs were separately tested. The possibility of inhibition by magnesium (Mg^{2+}) was tested using 30 mM magnesium sulfate (MgSO_4) in the assay buffer. In assessing the effect of freezing and cold storage on subsequent analysis of NR activity, *Enteromorpha* sp. tissue was rinsed in the field and samples frozen by immersion in liquid nitrogen. Replicates were frozen separately in 2-mL microcentrifuge tubes and stored at -20°C or -80°C . Sample FW was determined before freezing to avoid losses in NR activity due to release of proteolytic enzymes upon thawing. In the case where samples were frozen in the field, FW of the frozen tissue was quickly measured before extraction. It should be noted that after freezing with liquid nitrogen and storage at -80°C , *Enteromorpha* sp. FW was significantly lower ($P < 0.05$, paired samples t-test) and declined an average of $5.8 \pm 1.3\%$ (SE) ($n = 19$).

If existing NR is becoming activated or more NR is synthesized during the course of an *in situ* assay, then an *in situ* assay overestimates NR activity. Accordingly, an assessment of whether *in situ* assay conditions could lead to the induction or activation of NR activity was conducted. An *in vitro* assay was performed on replicate samples from a collection of *Enteromorpha* sp. tissue. Samples from the same collection were also assayed for NR activity using an *in situ* assay, but the algal tissue was removed from the assay buffer before ending the assay by heating. This tissue was then triple rinsed with deionized water and processed according to the *in vitro* assay protocol. The *in vitro* NR activity from the initial *in vitro* assay was then compared with the *in vitro* activity for the tissue exposed to the *in situ* assay but removed before heating.

Field application. To test the logistics of performing these assays in the field, the optimized *in situ* and *in vitro* assays were applied to stands of *Enteromorpha* sp. and *Gelidium* sp. at the collection site through 2000 and 2001. In addition, *in situ* NR activity and internal stores of NH_4^+ and NO_3^- were also measured 3 h after sunrise from 19 January until 27 January 2001 (9 days) for *Enteromorpha* sp. Internal stores of NH_4^+ and NO_3^- were extracted from 0.4 g FW homogenized in 45 mL of deionized water. The homogenate was boiled for 10 min, extracted for 24 h at 4° C, and filtered through a glass fiber filter (GF/C) before analysis for NH_4^+ and NO_3^- with a SanplusSystem autoanalyzer (SKALAR, Norcross, GA, USA) according to the protocol outlined in the SKALAR operations manual (SKALAR 1996). *In vitro* NR activity in *Enteromorpha* sp. was also measured over 24 h on 21 April 2000. During the 24-h sampling, *Enteromorpha* sp. tissue was collected and frozen every 4 h with two additional samples taken at the hour nearest sunrise and sunset. Samples were frozen at -80°C , and *in vitro* NR activity measured within 10 days of collection at the water temperature measured at the time of collection. Light level, salinity, temperature, and water column inorganic nitrogen levels were measured during both the 24-h and 9-day sampling regimes. Light level was measured using an LI-192SA underwater quantum sensor attached to an LI-1000 data logger (LI-COR, Lincoln, NE, USA) programmed to log mean light level over 5-min intervals. Salinity and temperature were measured with an Orion model 140 conductivity/temperature/salinity meter (Thermo Orion, Beverly, MA, USA). Water samples for nutrient analysis were filtered through a glass fiber filter (GF/C), frozen at -80°C , and later analyzed for NO_3^- and NH_4^+ with a SansplusSystems autoanalyzer.

Statistical analysis. Statistical comparisons were made using either a t-test for independent samples when two samples were compared or a one-way analysis of variance in conjunction with a Scheffe post-hoc test when comparing three or more samples. For sample sets that failed Levene's test for equality of variances, a Kruskal-Wallis test was performed in conjunction with a Games-Howell post hoc test (Sokal and Rohlf 1995, Underwood 1997). All correlations are Spearman correlations. All statistical comparisons were performed using SPSS Base 10.0 for windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Evaluation of short-term storage of fresh tissue on NR activity. The storage of fresh *Enteromorpha* sp. tissue led to a significant reduction in *in situ* NR activity over time ($P < 0.001$). For tissue that was stored between SW-moistened paper towels at 10° C in the dark, NR activity declined to $75.0 \pm 5.7\%$ and $51.1 \pm 7.1\%$ of the initial value after 1 and 2.5 h, respectively (mean \pm SE). For the tissue stored in 10° C SW in the dark, NR activity was $45.3 \pm 3.9\%$ and $69.9 \pm 5.5\%$ of the initial value after 1 and 2.5 h, respectively (mean \pm SE).

Assay optimizations. The *in situ* assay was optimized for *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. However, when all three species responded similarly to the test conditions, the results for only one species are reported with NR activity presented as the mean percentage \pm SE relative to the highest value. The results for the optimization of the *in vitro* assay on *Enteromorpha* sp. and *Gelidium* sp. are reported in the same manner. One hundred percent NR activity corresponds to roughly $36 \times 10^{-3} \text{ U} \cdot \text{g}^{-1} \text{ FW}$ for the *in situ* assay and $20 \times 10^{-3} \text{ U} \cdot \text{g}^{-1} \text{ FW}$ for the *in vitro* assay ($\text{U} = \mu\text{mol NO}_3^- \text{ reduced} \cdot \text{min}^{-1}$).

In situ NR assay. Assays performed in 100 mM phosphate buffer showed reduced activity in comparison with ASW (ASW = $100 \pm 4.5\%$, 100 mM phos-

phate buffer = $60.3 \pm 3.1\%$, *Enteromorpha* sp., $n = 3$, $P < 0.001$), whereas filtered seawater (FSW) and ASW produced similar activity (ASW = $100 \pm 4.5\%$, FSW = $93.2 \pm 2.7\%$, *Ulva* sp., $n = 4$, $P > 0.05$). A salinity of 20 psu produced the highest mean activity (Table 1). On average the salinity at the collection site during the 2000 growing season was 21.5 ± 6.9 psu (SD, $n = 7109$) and was 25.1 psu when the test for optimal salinity was performed. A pH of 8.22, the unadjusted pH of the ASW, produced the highest activity (Table 1).

The presence of *n*-propanol in the assay buffer led to higher NR activity up to a point (Table 1). Beyond 2% to 3% *n*-propanol, however, activity levels declined. The optimal *n*-propanol concentration for *Enteromorpha* sp. and *Ulva* sp. was 2.25% and for *Gelidium* sp. was 3%. The substitution of DMSO for *n*-propanol resulted in lower activity over the range of DMSO concentrations tested (Table 1).

The addition of 0.2 mM NADH to the assay buffer did not produce a significant change in NR activity (no NADH = $100.0 \pm 9.3\%$, 0.2 mM NADH = $86.9 \pm 13.5\%$, *Gelidium* sp., $n = 5$, $P > 0.05$). NR activity did increase significantly when NO_3^- concentration increased from 0 to 30 mM, with no significant difference between NO_3^- concentrations over 30 mM (Table 1).

In vitro activities measured for tissue before an *in situ* assay ($13.88 \pm 1.41 \times 10^{-3} \text{ U} \cdot \text{g}^{-1} \text{ FW}$) and for tissue at the end of an *in situ* assay, just before immersion in $>80^\circ \text{C}$ water ($18.25 \pm 2.78 \times 10^{-3} \text{ U} \cdot \text{g}^{-1} \text{ FW}$) were not significantly different ($n = 3$, $P > 0.05$). Anaerobic conditions did not appear to be necessary to produce the highest level of NR activity in macroalgae (aerobic = $100.0 \pm 0.6\%$, anaerobic = $99.6 \pm 6.5\%$, *Enteromorpha* sp., $n = 3$, $P > 0.05$).

Nitrite production during the *in situ* assay was linear over the initial 60 min. After 60 min, NO_2^- production began to gradually decline. As the assay proceeded past 60 min, the variability among samples began to increase as well. Ending the assay at 60 min minimized this variability and avoided loss of NR activity. Nitrite uptake occurred when NO_3^- was replaced with NO_2^- in the *in situ* assay buffer with *Enteromorpha* sp. (Table 2). This uptake of NO_2^- was reduced when *n*-propanol was present.

TABLE 1. Percentage of NR activity for different *in situ* assay conditions (means \pm SE).

Condition	Species	% of NR activity	n
0 psu	<i>Gelidium</i> sp.	-8.5 ± 1.6 a	3
10 psu		6.2 ± 7.3 a	3
20 psu		100.0 ± 1.5 b	3
30 psu		26.1 ± 28.9 a,b	2
pH 6.42	<i>Ulva</i> sp.	14.9 ± 1.8 a	4
pH 6.98		18.8 ± 1.8 a	4
pH 7.58		19.4 ± 1.3 a	4
pH 8.22		100.0 ± 16.1 a	3
pH 8.86		33.7 ± 9.2 a	4
0% <i>n</i> -propanol		<i>Gelidium</i> sp.	0.4 ± 0.8 a
1%	23.6 ± 0.7 b		3
3%	100.0 ± 7.1 c		3
5%	7.1 ± 0.7 d		3
0% <i>n</i> -propanol	<i>Enteromorpha</i> sp.		27.8 ± 8.8 a
1%		35.0 ± 3.0 a	3
2%		100.0 ± 14.1 b,c	3
3%		88.2 ± 5.2 b,c	3
4%		39.4 ± 8.7 a,c	3
5%		20.7 ± 9.8 a	3
0.1% DMSO		<i>Enteromorpha</i> sp.	33.3 ± 6.2 a
0.5%	24.0 ± 2.0 a		3
1%	19.0 ± 2.1 a		3
3%	22.9 ± 1.3 a		3
1% <i>n</i> -propanol	<i>Enteromorpha</i> sp.	100.0 ± 4.2 b	3
0 mM KNO_3		26.7 ± 2.7 a	4
5 mM		28.8 ± 2.9 a	4
10 mM		38.4 ± 5.5 a	4
30 mM		71.4 ± 10.6 b	4
50 mM		91.7 ± 9.7 b	4
100 mM	100.0 ± 5.1 b	4	

Assays were run for 1 h at 30° C. When the response of all three species (*Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp.) to the conditions tested resembled one another, the results for only one species are shown. Treatments that share letters are not significantly different at the 95% confidence interval.

TABLE 2. Nitrite uptake for *Enteromorpha* sp. expressed in $\mu\text{mol NO}_2^- \cdot \text{g}^{-1} \text{ FW} \cdot \text{h}^{-1}$ measured over 1 h in 20 psu ASW with and without 2.25% *n*-propanol at 25° C (means \pm SE, $n = 4$).

Initial NO_2^- addition (μM)	Uptake rate ($\mu\text{mol NO}_2^- \cdot \text{g}^{-1} \text{ FW} \cdot \text{h}^{-1}$)	
	2.25% <i>n</i> -propanol	No <i>n</i> -propanol
0	-0.01 ± 0.00	—
5	—	0.17 ± 0.01
10	0.12 ± 0.01	0.32 ± 0.02
50	0.45 ± 0.03	1.39 ± 0.11
200	0.72 ± 0.03	—

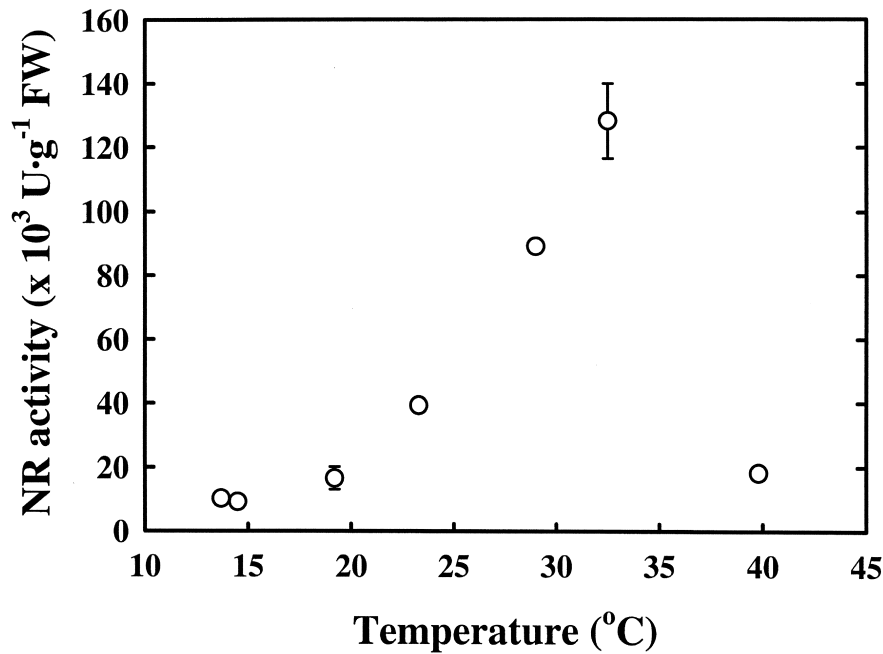


FIG. 1. *In situ* NR activities for *Enteromorpha* sp. incubated over a range of temperatures. Points are the mean values \pm SE ($n = 3$). Error bars are smaller than symbols if not visible.

The effect of temperature on *in situ* NR activity was nonlinear (Fig. 1). NR activity increased from 13.7 to 32.5° C with a 9-fold increase between 32.5 and 39.8° C. These results indicated a temperature optima around 32° C for NR in *Enteromorpha* sp. Temperature at the collection site annually ranges from 4 to 34° C and was 22.1° C when this experiment was conducted.

Although heating samples to >80° C is an effective way to inactivate NR and end the *in situ* assay, the addition of 1.2 N HCl was tested as a possible alternative that would be easier to apply in the field. However, heat-based termination of the *in situ* assays yielded consistently higher estimates of NR activity than termination with acid addition.

In vitro NR assay. Although the addition of casein to the extraction buffer led to higher *in vitro* activity, the addition of BSA to the extraction buffer did not enhance activity (Table 3). Triton X-100, a non-ionic detergent, and PVP, a compound that binds phenolics, produced no significant increase in NR activity when added to the *in vitro* extraction buffer (*Enteromorpha* sp. [no Triton X-100 = 100.0 \pm 16.8%, 0.1% Triton X-100 = 70.7 \pm 19.9%, $n = 4$, $P > 0.05$; no PVP = 100.0 \pm 8.4%, 0.3% PVP = 87.2 \pm 4.7, $n = 4$, $P > 0.05$], *Gelidium* sp. [no Triton X-100 = 70.1 \pm 13.3%, 0.1% Triton X-100 = 100.0 \pm 23.2%, $n = 4$, $P > 0.05$; no PVP = 100.0 \pm 19.0%, 0.3% PVP = 17.8 \pm 21.6%, $n = 4$, $P < 0.05$]). In the case of PVP, its presence actually led to a significant decrease in activity for *Gelidium* sp.. The presence of millimolar concentrations of Mg²⁺ in the extraction buffer led to a significant reduction in NR activity (no MgSO₄ = 100.00 \pm 3.0%, 30 mM MgSO₄ = 56.1 \pm 5.3%, *Enteromorpha* sp., $n = 3$, $P < 0.01$).

The use of a mortar, pestle, and liquid nitrogen during extraction produced higher activity than the homogenization of the algal tissue with an OMNI 2000 tissue homogenizer (liquid nitrogen = 100.0 \pm 11.9%, homogenization = 46.8 \pm 1.2%, *Enteromorpha* sp., $n = 3$, $P < 0.01$). Most algal cells were ruptured when ground in liquid nitrogen, but not when homogenized (visualized via microscopy). Centrifuging the extract before its introduction into the assay buffer led to a 25% reduction in activity (centrifuged = 75.5 \pm 1.5%, not centrifuged = 100.0 \pm 2.7%, *Enteromorpha* sp., $n = 3$, $P < 0.01$).

The optimal pH for the *in vitro* assay buffer was roughly 8.0 (Table 3). In the assay buffer, substituting Tris buffer pH 8.0 (buffering capacity pH 7 to 9) for phosphate buffer pH 8.0 (buffering capacity pH 6.5 to 7.5) led to reduced activity (100 mM phosphate buffer = 100.00 \pm 11.9%, 100 mM Tris buffer = 33.5 \pm 1.0%, *Enteromorpha* sp., $n = 3$, $P < 0.01$). During the optimization of the *in vitro* assay buffer, NR activity in *Enteromorpha* sp. appeared to level off between 0.05 and 0.1 mM NADH (Table 3).

Evaluation of frozen sample storage. The optimized *in vitro* NR assay detected NR activity in *Ulva* sp. and *Enteromorpha* and *Gelidium* sp. (Table 4). This activity was preserved over time for samples of *Enteromorpha* sp. immediately frozen in liquid nitrogen and stored at -80° C ($P > 0.05$) but not for samples stored at -20° C ($P < 0.001$, Fig. 2). The variability on day 25 for samples stored at -80° C was high. However, because the mean value for that day (15.93 \pm 2.24 10^{-3} U·g⁻¹ FW) was higher than the mean value for day 0 (10.68 \pm 0.35 10^{-3} U·g⁻¹ FW), the variability on day 25 is likely due to random variation rather than degradation.

TABLE 3. Percentage of NR activity for different *in vitro* assay conditions (means \pm SE).

Condition	Species	% of NR activity	n
Extraction buffer			
2% casein	<i>Enteromorpha</i> sp.	100.0 \pm 1.1 a	3
2% BSA		37.7 \pm 1.5 b	3
No protein added		51.0 \pm 0.1 c	3
Assay buffer			
pH 7.21	<i>Enteromorpha</i> sp.	45.1 \pm 2.6 a	3
pH 7.51		52.4 \pm 5.5 a	3
pH 7.75		85.9 \pm 4.2 b	3
pH 7.98		94.2 \pm 0.8 b	3
pH 8.23		100.0 \pm 1.5 b	3
0.025 mM NADH	<i>Enteromorpha</i> sp.	85.2 \pm 3.0 a	3
0.05 mM NADH		88.8 \pm 1.5 a, b	3
0.1 mM NADH		96.7 \pm 1.6 a, b	3
0.2 mM NADH		100.0 \pm 4.0 b	3

Assays were run for 30 min at 30° C. The *in vitro* assay conditions were optimized for *Enteromorpha* sp. and *Gelidium* sp. When the response of the two species to the conditions tested resembled one another, the results for only *Enteromorpha* sp. are shown. Treatments that share letters are not significantly different at 95% confidence limit.

NR activity in the field. Using the *in situ* and *in vitro* methods, NR activity was consistently found in *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. when sampled during 2000 and 2001 (see Table 4 for representative values). *In situ* activity was consistently greater than *in vitro* activity throughout the course of this study (Table 4).

The range of *in situ* and *in vitro* NR activity values for *Enteromorpha* sp. monitored over 9 days and 24 h, respectively, demonstrated the variability in NR activity exhibited in the field (Figs. 3A and 4). *In situ* NR activity varied 7-fold over the 9 days, whereas *in vitro* NR activity varied nearly 5-fold over 24 h. The difference of a few days or even a few hours can make a substantial difference in water column nutrient concentrations or light levels (Figs. 3, C and D and 4).

Over the 9-day period, the most substantial change in NR activity occurred after a decline in salinity from 20.6 to 14.5 psu (Fig. 3, A and B). This drop in salinity coincided with an increase in water column NO₃⁻ and a decrease in water column NH₄⁺ (Fig. 3, C and D). In general, when NO₃⁻ concentrations were low and NH₄⁺ concentrations were high, NR activity tended to remain low (<8 \times 10⁻³ U·g⁻¹ FW) and vice versa. Al-

though internal stores of NH₄⁺ remained low over the 9 days (Fig. 3D), internal stores of NO₃⁻ began increasing 1 day after the initial increase in water column NO₃⁻ concentration (Fig. 3C). The ambient temperature dropped from 12.8° C to 3.4° C over the first 3 days before gradually increasing back to 13.0° C over the remaining 6 days (Fig. 3B). Light levels at the time of algal collection varied between 1900 and 2400 μ mol photons·m⁻²·s⁻¹, with the exception of 19 and 27 January, when light levels dropped to \sim 200 μ mol photons·m⁻²·s⁻¹.

NR activity was significantly correlated to water column NO₃⁻ (0.733, *P* < 0.05), water column NH₄⁺ (-0.783, *P* < 0.05), temperature (0.800, *P* < 0.05), and salinity (-0.753, *P* < 0.05) but was not significantly correlated to internal stores of inorganic nitrogen or light (Spearman's correlation coefficient). The increase in NO₃⁻ concentration and decline in NH₄⁺ concentration that accompanies a drop in salinity, rather than a direct effect of salinity on NO₃⁻ metabolism, likely drives the strong negative correlation between NR activity and salinity.

The use of the *in vitro* assay to follow NR activity in a field population of *Enteromorpha* sp. suggests that light levels can influence diel patterns in NR activity (Fig. 4). Other than light level, the environmental parameters measured for the 24-h period remained fairly constant (temperature = 22.6 \pm 0.4° C [*n* = 12], [NO₃⁻] = 2.68 \pm 1.3 μ M [*n* = 3], [NH₄⁺] = 2.9 \pm 0.6 [*n* = 3], and salinity = 5.5 \pm 0.1 psu [*n* = 12]).

DISCUSSION

Because the storage and transport of fresh tissue leads to a substantial reduction in NR activity, it is necessary to measure NR activity on site or on tissue frozen on site if the goal is to capture field levels of NR activity. The *in situ* assay presented here is simple and can be performed on site. The demonstration that algal tissue can be frozen in liquid nitrogen and enzymatic activity preserved by storage at -80° C verifies that the *in vitro* assay we present can also be used to measure levels of enzyme activity from tissue frozen on site.

In situ NR assay. In its combined use of ASW and *n*-propanol, the optimized *in situ* assay presented here resembles the *in situ* assay previously published by Davison and Stewart (1984a,b) for use with *Laminaria*

TABLE 4. Representative NR activity values expressed in U·g⁻¹ FW for *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. measured in the field using the optimized *in situ* and *in vitro* assays (means \pm SE; U = μ mol NO₃⁻ reduced·min⁻¹).

Species	NR activity (10 ³ U·g ⁻¹ FW)	n	Month	Salinity (psu)	Temperature (° C)
<i>In situ</i> assay					
<i>Enteromorpha</i> sp.	19.33 \pm 0.83	8	Jan.	14.5	9.5
<i>Ulva</i> sp.	27.50 \pm 2.67	5	Oct.	28.1	24.0
<i>Gelidium</i> sp.	23.83 \pm 2.50	8	Mar.	2.6	13.7
<i>In vitro</i> assay					
<i>Enteromorpha</i> sp.	9.67 \pm 1.17	3	Feb.	24.7	19.5
<i>Ulva</i> sp.	3.67 \pm 0.17	3	Oct.	28.1	24.1
<i>Gelidium</i> sp.	11.50 \pm 0.33	3	Feb.	24.7	19.5

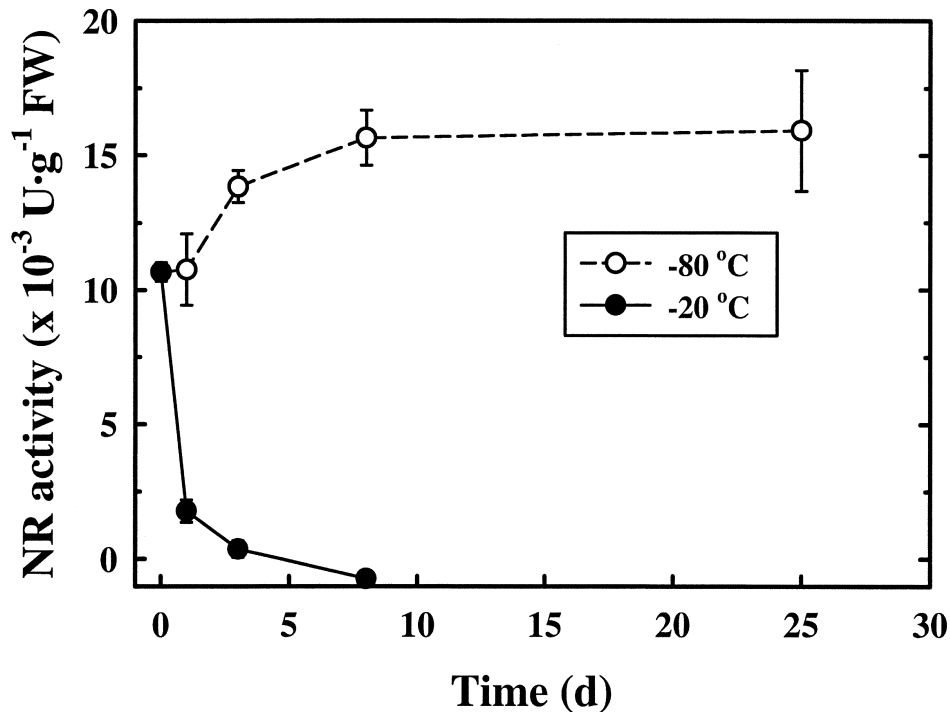


FIG. 2. *In vitro* NR activity for *Enteromorpha* sp. after storage at -20°C or -80°C . Points are mean values \pm SE ($n = 3$).

ans. Although phosphate buffer and FSW have been presented as alternatives to ASW (Dipierro et al. 1977, Corzo and Neill 1991), assays performed in phosphate buffer, as part of this study, showed reduced activity in comparison with ASW. In our work, FSW and ASW produced similar activity, but concerns about differences in FSW collected at different times led to the selection of ASW for the optimized assay.

Although the addition of NO_3^- to the *in situ* assay buffer increases NR activity, NO_3^- can be excluded from the assay buffer and NR activity still detected. However, it is not clear that either no NO_3^- or NO_3^- added to saturation reflects conditions that exist within intact nonpermeabilized algal cells. We chose to include NO_3^- at a saturating concentration because the highest proportion of NR activity is detected at a saturating concentration and differences between treatments are more likely to be detected when this maximal activity is measured.

It is important to point out that the addition of NO_3^- to the assay buffer is problematic if the exogenous NO_3^- is leading to the induction or activation of NR during the *in situ* assay. If the activation of existing NR or the synthesis of additional NR occurred, then the *in situ* assay would be overestimating activity. The similarity between *in vitro* activities measured for tissue before an *in situ* assay ($13.88 \pm 1.41 \times 10^{-3} \text{ U}\cdot\text{g}^{-1} \text{ FW}$) and for tissue at the end of an *in situ* assay, just before immersion in $>80^{\circ}\text{C}$ water ($18.25 \pm 2.78 \times 10^{-3} \text{ U}\cdot\text{g}^{-1} \text{ FW}$) suggests that neither the synthesis/activation nor the degradation/deactivation of NR

likely occurred during our *in situ* assay. Because the cellular machinery is disrupted during the extraction step in an *in vitro* assay, *in vitro* assays provide a snapshot of NR activity. Although the cellular machinery is not disrupted to the same extent during an *in situ* assay, it appears that the cells are at least unable to alter levels of active NR in *Enteromorpha* sp. Thomas and Harrison (1988), on the other hand, found that *in vitro* NR activity measured during an *in situ* assay changed over time, indicating that levels of active NR were being altered in *Porphyra perforata*, *Enteromorpha intestinalis*, and *Fucus gardneri* during the course of an *in situ* assay. As a result, we recommended that for each species a comparison of *in vitro* activity during the course of an *in situ* assay should be made before using an *in situ* assay to answer questions about changes in NR activity.

Based on work in higher plants (Canvin and Woo 1979), it has been suggested that anaerobic conditions are required during *in situ* assays to obtain the highest possible NR activity in macroalgae (Corzo and Neill 1991). In spinach leaves, the highest activity occurred under anaerobic conditions in the absence of alcohol (Corzo and Neill 1991). *In situ* NR activity was detected in the presence of oxygen, but only when an alcohol had been included in the assay buffer (Canvin and Woo 1979). This inhibition of NO_3^- reduction in the dark by oxygen was attributed to competition for cytosolic NADH between NO_3^- reduction and dark respiration (Canvin and Woo 1979, Beevers and Hageman 1980, Reed and Canvin 1982, Maurino et al.

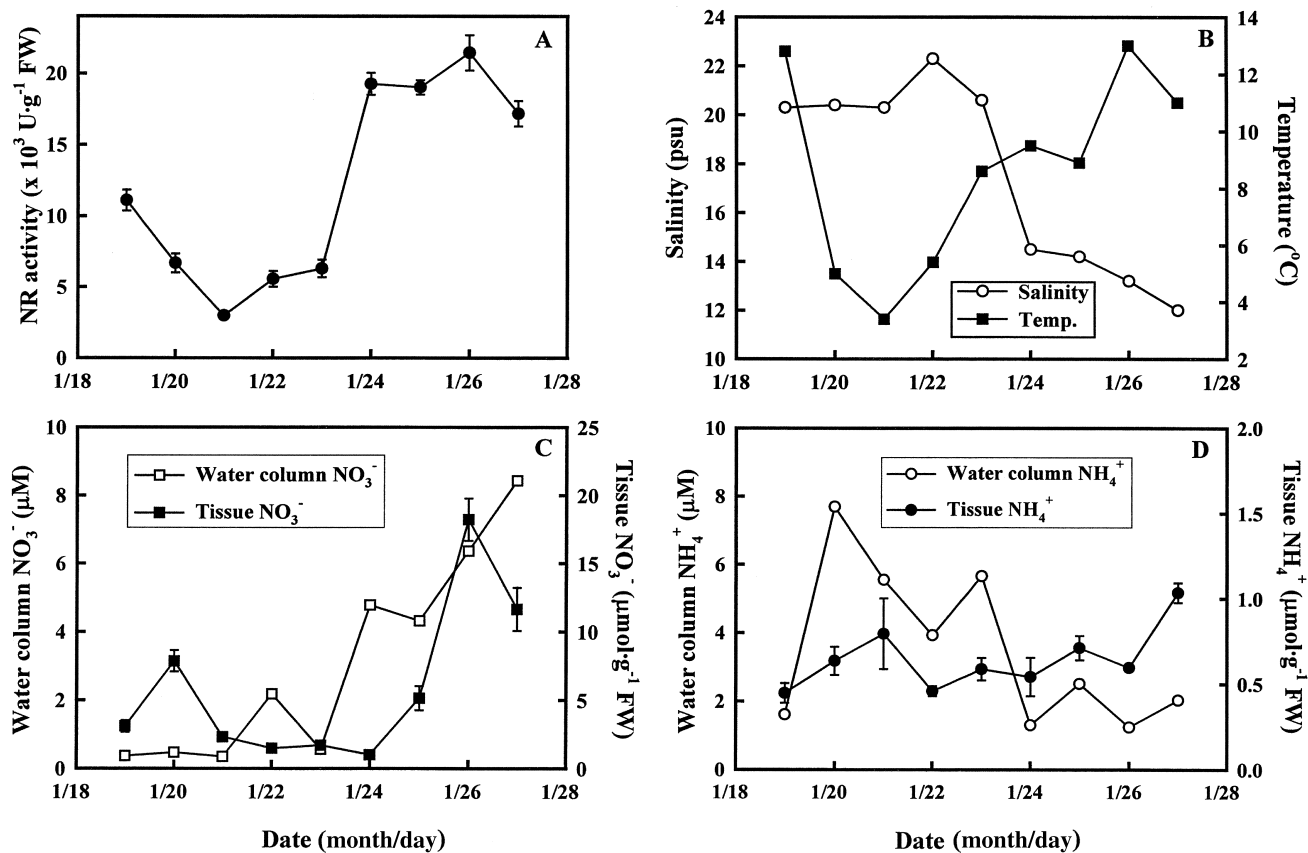


FIG. 3. (A) *In situ* NR activity for *Enteromorpha* sp. NR values are mean values \pm SE ($n = 8$). (B) Salinity and temperature. (C) Water column NO_3^- and tissue NO_3^- for *Enteromorpha* sp. (mean values \pm SE, $n = 5$). (D) Water column NH_4^+ and tissue NH_4^+ for *Enteromorpha* sp. (mean values \pm SE, $n = 5$). All measurements taken 3 h after sunrise from 19 to 27 January 2001.

1985). The inhibitory effects of oxygen were not apparent for the three macroalgal species tested here. Two possible explanations are that macroalgal NR has a much higher affinity for NADH in comparison with the enzymes of dark respiration or oxygen levels and rates of respiration in the dark are lower in macroalgae than in vascular plants.

Brinkhuis et al. (1989) raised the possibility that substantial NO_2^- uptake may occur during *in situ* NR assays. In this study, when NO_2^- was substituted for NO_3^- in the *in situ* assay buffer, measurable NO_2^- uptake occurred with *Enteromorpha* sp. The presence of *n*-propanol reduced the amount of uptake but did not eliminate it. The highest *in situ* NR activity measured during this study at 25°C was $88.67 \pm 0.50 \times 10^{-3} \text{ U}\cdot\text{g}^{-1} \text{ FW}$, which equates to a final NO_2^- concentration of $81 \mu\text{M}$ in the sample vial. A more typical *in situ* NR activity value of $20.33 \pm 3.50 \times 10^{-3} \text{ U}\cdot\text{g}^{-1} \text{ FW}$ equates to a final NO_2^- concentration of $25 \mu\text{M}$. Nitrite uptake at a starting concentration of $25 \mu\text{M}$ would be roughly $0.25 \mu\text{mol NO}_2^- \cdot \text{g}^{-1} \text{ FW}\cdot\text{h}^{-1}$. This uptake rate is approximately 20% of a typical *in situ* NR activity. Hence, if NO_2^- uptake occurs as seen here during an *in situ* assay, a conservative estimate of the underestimation of NR activity due to NO_2^- up-

take would be 20%. Nitrite uptake in the presence of NO_3^- was not tested here. It is possible that NO_2^- uptake would be reduced in the presence of NO_3^- because internal NO_2^- concentrations would be gradually increasing with the internal reduction of NO_3^- to NO_2^- . This gradual increase in internal NO_2^- would reduce the passive movement of NO_2^- into the cells and so minimize reentry of NO_2^- into the cytosol. Because, NO_2^- reduction occurs in the chloroplast and is thought to require an intact and active plastid (Crawford 1995), NO_2^- reduction is likely not a factor in modifying NO_2^- flux across the cell membrane.

The dependence of *in situ* NR activity on temperature indicates that ambient water temperatures may have a substantial impact on NR activity in the field. Levels of NR activity may vary significantly, particularly for macroalgae that experience large shifts in temperature diurnally or seasonally. Kristiansen (1983) and Gao et al. (2000) found a similar response to temperature for cultured and natural communities of phytoplankton. The 9-fold decrease in NR activity between 32.5 and 39.8°C indicates that intertidal *Enteromorpha* sp. may be temperature stressed during late summer when water temperatures in shallow near-shore environments can exceed 30°C and air temper-

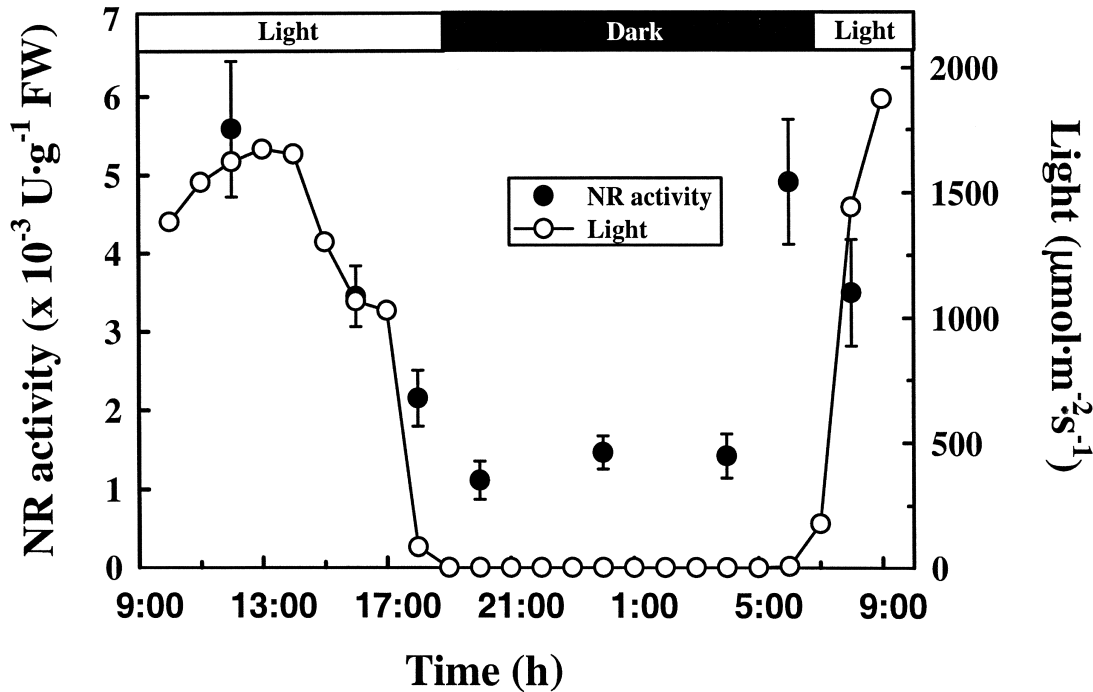


FIG. 4. Light levels and *in vitro* NR activity for *Enteromorpha* sp. over 24 h on 21 and 22 April 2000. NR values are mean values \pm SE ($n = 5$).

atures can reach 39° C. Coincidentally, *Enteromorpha* sp. often disappears from the Northern Gulf of Mexico during late summer (Kaldy et al. 1995).

As mentioned above, the three species responded similarly to the different *in situ* assay conditions tested with the exception of the percentage of *n*-propanol. If the application of this assay to other macroalgal species results in little or no measurable NR activity, we recommend adjusting this component of the assay buffer. Because of the possibility of species-specific responses to the *in situ* assay buffer, particularly the permeabilizing agent, comparisons of NR activity levels between the three species sampled in this study were not made, and in general such comparisons are problematic. However, differences in the percent change in NR activity for two species in response to distinct treatments (e.g. light level) could be used as an indicator of more rapid induction or activation of NR in one species.

In vitro NR assay. Our optimized *in vitro* NR assay differs from past *in vitro* assays used on macroalgae in its use of casein, cysteine, KNO_3 , and FAD in the extraction buffer (Thomas and Harrison 1988, Hurd et al. 1995). The addition of bulk protein provides a high protein concentration, which may stabilize extracted enzymes and/or serve as substrate for cellular proteases, decreasing the possibility that these proteases can bind and degrade enzymes. Although the addition of casein led to higher *in vitro* activity, the addition of BSA to the extraction buffer in this study and in Hurd et al. (1995) did not enhance activity, so the stabilizing effect of casein may be particular to NR. The addition

of cysteine to the extraction buffer was motivated by its use in higher plant assays to protect thiol groups on NR from oxidation (Maurino et al. 1985). Hurd et al. (1995) used dithiothreitol in a similar role. Since the work of Pistorius et al. (1976) on *Chlorella* indicated that the presence of NO_3^- may be necessary to maintain NR in its active state, NO_3^- was added to the extraction buffer as well. Similarly, the addition of FAD to the extraction buffer was based on enhancement of NR activity in the presence of FAD in some species of microalgae (Vennesland and Solomonson 1972, Berges and Harrison 1995). In certain systems, FAD may be loosely bound to NR and capable of disassociating from the enzyme during extraction (Berges 1997). In Berges and Harrison (1995) and in this study, substituting Tris buffer for phosphate buffer led to reduced activity, suggesting that the ionic effect, in addition to the buffering capacity of the extraction and assay mediums, may be important.

The reduction in NR activity with the addition of PVP to the extraction buffer noted by Hurd et al. (1995) for *Fucus gardneri* was similar to the decline in activity noted in this work for *Gelidium* sp. Although NR activity was not significantly changed by the presence of Triton X-100, its addition to the extraction buffer may be advisable, especially for investigators who initially have difficulty detecting NR activity when applying this assay to other species. A similar approach was used by Berges and Harrison (1995), who included Triton X-100 in their optimized assay after finding that whereas it did not increase NR activity, it did eliminate NR activity in the pellet produced

when their microalgal extract was centrifuged before being assayed.

The inactivation of NR is now thought to be a two-step process involving the phosphorylation of NR followed by the binding of a 14-3-3 NR inhibitory protein (NIP) (Chung et al. 1999, Finnie et al. 1999). This second step requires Mg^{2+} (Chung et al. 1999). In this study, the presence of millimolar concentrations of Mg^{2+} in the *in vitro* extraction buffer led to a significant reduction in NR activity. This result suggests that active forms of NR from *Enteromorpha* and *Gelidium* sp. may be phosphorylated and that dephosphorylation followed by the binding of NIP during the *in vitro* assay could explain low or undetectable levels of NR activity.

Comparison of in situ versus in vitro assay. *In situ* activity was consistently greater than *in vitro* activity throughout the course of this study. This difference likely reflects the degradation or inactivation of NR during the extraction step in the *in vitro* assay. Inadequate breakage of cells during grinding is probably not a factor because inspection of the extracted tissue under magnification ($\times 1000$) revealed that nearly all the algal cells had been ruptured and their contents released. Although the use of an optimized *in vitro* extraction buffer can reduce the amount of degradation and inactivation, it does not eliminate it entirely. However, the use of the *in vitro* assay allows for samples to be frozen and then processed at a more convenient time and, thus, is advised when a large number of samples must be collected simultaneously. In addition, if K_m and V_{max} values are sought, the controlled conditions of the *in vitro* assay make it the only suitable choice. The relationship between *in situ* and *in vitro* values found here for *Enteromorpha* sp., *Ulva* sp., and *Gelidium* sp. may not hold for macroalgae with thicker thalli, which may be more resistant to permeabilization by *n*-propanol.

NR activity in the field. The representative *in situ* NR activity of $19.33 \pm 0.83 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW we report for *Enteromorpha* sp. is considerably higher than the $0.8 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW found by Thomas and Harrison (1988) for *Enteromorpha intestinalis* wrapped in plastic wrap and stored (1–2 h) before being assayed (using $10 \text{ mg protein}\cdot\text{g}^{-1}$ FW conversion from Thomas [1983] suggested by Hurd et al. [1995]) and the $4.2 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW reported by Thomas and Harrison (1988) for *E. intestinalis* preincubated in $30 \mu\text{M NO}_3^-$. The $13.8 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW reported by Maier and Pregnall (1990) for *E. intestinalis* is closer to the representative value reported in this study. However, the *in vitro* NR value of $26.7 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW measured at room temperature for *E. intestinalis* found by Thomas and Harrison (1988) is nearly 3-fold higher than the representative value of $9.67 \pm 1.17 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW reported here. Although *in vitro* NR activity values as high as $55 \pm 4.5 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW were found during our investigations, the higher values were either for assays performed on tissue exposed to high light and high NO_3^- concentrations in the field or for assays conducted in the lab at temperatures between 25 and 30°C.

The $27.50 \pm 2.67 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW we report for *in situ* NR activity of *Ulva* sp. is higher than many of the values reported by other investigators for *Ulva*, $0.42 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW (Murthy et al. 1986), $3.8 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW (Corzo and Neill 1991), and $5.9 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW (Gao et al. 1992), but similar to the $29.3 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW reported by Gao et al. (1992). The representative *in vitro* NR activity of $3.67 \pm 0.17 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW found in this study is lower than the $9.3 \pm 1.6 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW reported in the literature by Hurd et al. (1995).

NR activity for *Gelidium* sp. has not been previously published, but the *in situ* and *in vitro* values reported here are similar to those reported in the literature for other Rhodophyceae. Literature *in situ* NR activity values for Rhodophyceae range from $0.4 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW for *Petroglossum nicaeense* (Dipierro et al. 1977) to $158 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW for *Porphyra umbicalis* (Hernandez et al. 1993). For *in vitro* NR activity of Rhodophyceae, the literature reports a range from $4.0 \pm 0.96 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW for *Porphyra* sp. (Hurd et al. 1995) to $33.2 \times 10^{-3} \text{ U}\cdot\text{g}^{-1}$ FW for *Porphyra yezoensis* (Araki et al. 1979).

Several factors, such as field conditions when algae are collected, assay temperature, and treatment of the algae before assay, can influence NR activity and make comparison between literature values problematic. For example, the range of *in situ* and *in vitro* NR activity values for *Enteromorpha* sp. monitored over 9 days and 24 h, respectively, demonstrate the variability in NR activity observed in the field. The difference of a few days or even a few hours can make a substantial difference in the environmental conditions and, consequently, NR activity levels.

While prior studies have measured seasonal differences in NR activity in macroalgae (Davison et al. 1984, Hurd et al. 1995), no study has measured differences in NR activity for field populations without transporting the samples or holding the samples for a period of time before assays were performed. Moreover, no study has followed water column nutrient concentrations, internal stores of inorganic nitrogen, and NR activity over daily intervals in the field.

Overall, the patterns observed during our 9-day field sampling indicate that NR activity in *Enteromorpha* sp. is highly responsive to daily fluctuations in NO_3^- and NH_4^+ availability and temperature when light levels are similar. However, conclusions based on this study are limited by the small size of the data set. A more comprehensive field study that encompasses more than one season is needed before conclusions on the role of environmental and physiological parameters in predicting NR activity can be broadened.

Several studies have documented the inhibition of NO_3^- reduction and uptake by NH_4^+ in marine algae (e.g. Lund 1987, Riccardi and Solidoro 1996, Varela and Harrison 1999). The strong positive correlation between NR activity and water column NO_3^- and the strong negative correlation between NR activity and water column NH_4^+ suggests that NH_4^+ inhibition of

NO_3^- reduction likely occurs in the field as well. However, the presence of appreciable NR activity under conditions of low NO_3^- availability ($<0.5 \mu\text{M}$) and relatively high NH_4^+ concentration ($>4 \mu\text{M}$) suggests that NH_4^+ inhibition may not be complete and that levels of active NR may remain elevated to reduce internal stores of NO_3^- or take advantage of rapidly fluctuating NO_3^- loads.

The use of the *in vitro* assay to follow NR activity in a field population of *Enteromorpha* sp. suggests that light levels can influence diel patterns in NR activity. Because the environmental parameters other than light measured for the 24-h period remained fairly constant, the diel pattern in NR activity was attributed to the change in light level. The pattern observed in this study is reminiscent of the pattern seen by Gao et al. (1992) for *Ulva fenestrata* and Lopes et al. (1997) for *Gracilaria tenuistipitata*. However, it is important to note that Gao et al. (1992) and Lopes et al. (1997) conducted their experiments in the laboratory under light:dark cycles where light levels remained constant during the light cycle.

Conclusions. The *in situ* and *in vitro* NR assays presented here allow investigations of NO_3^- metabolism in *Enteromorpha* sp., *Ulva* sp., *Gelidium* sp., and possibly other species of macroalgae to be moved into the field. The use of liquid nitrogen to freeze algal samples before the use of an *in vitro* assay provides a reliable technique for preserving NR activity and likely the activity of other enzymes for later analysis. The application of these two assays on *Enteromorpha* sp. demonstrates that NR activity in the field responded to NO_3^- and NH_4^+ availability and was dependent on photoperiod. Together these two assays provide researchers with a reliable tool for investigating the interaction between environmental signals (e.g. light levels) and physiological signals (e.g. metabolite levels) on macroalgal NO_3^- reduction in the field.

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