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DISTINCT PATTERNS OF NITRATE REDUCTASE ACTIVITY IN BROWN ALGAE: LIGHT AND AMMONIUM SENSITIVITY IN *LAMINARIA DIGITATA* IS ABSENT IN *FUCUS* SPECIES[1]

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***Fucus* and *Laminaria* species, dominant seaweeds in the intertidal and subtidal zones of the temperate North Atlantic, experience tidal cycles that are not synchronized with light:dark (L:D) cycles. To investigate how nutrient assimilation is affected by light cycles, the activity of nitrate reductase (NR) was examined in thalli incubated in outdoor tanks with flowing seawater and natural L:D cycles. NR activity in *Laminaria digitata* (Huds.) Lamour. showed strong diel patterns with low activities in darkness and peak activities near midday. This diel pattern was controlled by light but not by a circadian rhythm. In contrast, there was no diel variation in NR activity in *Fucus serratus* L., *F. vesiculosus* (L.) Lamour., and *F. spiralis* L. either collected directly from the shore or maintained in the outdoor tanks. In laboratory cultures, transfer to continuous darkness suppressed NR activity in *L. digitata*, but not in *F. vesiculosus*; continuous light increased NR activity in *L. digitata* but decreased activity in *F. vesiculosus*. Furthermore, 4 d enrichment with ammonium (50 $\mu mol \cdot L^{-1}$ pulses), resulted in NR activity declining by >80% in *L. digitata*, but no significant changes in *F. serratus*. Seasonal differences in maximum NR activity were present in both genera with activities highest in late winter and lowest in summer. This is the first report of NR activity in any alga that is not strongly regulated by light and ammonium. Because light and tidal emersion do not always coincide, *Fucus* species may have lost the regulation of NR by light that has been observed in other algae and higher plants.**

Key index words: **ammonium; diel; intertidal; light; nitrate reductase**

Abbreviations: **ANOVA, analysis of variance; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NR, nitrate reductase**

Brown macroalgae dominate coastal algal biomass and account for the majority of the primary production in temperate coastal ecosystems. Productivity in these ecosystems is typically limited by nitrate supply (Dugdale 1967), and thus the capacity of macroalgae to take up and assimilate nitrogen (N) is critical to energy and nutrient cycling in coastal environments.

Macroalgae growing in the intertidal zone of many temperate regions are challenged with daily cycles of emersion and immersion. When exposed to the air, light availability is good, but the thalli are separated from sources of dissolved nutrients. When immersed by the incoming tide, dissolved nutrients are replenished, but water depth may reduce light availability. Moreover, as tidal cycles shift, the coincidence of emersion and light also changes. Intertidal macroalgae thus have a unique need to balance resource acquisition, particularly that of N, carbon (C), and energy (light). Uptake and assimilation of N in algae strongly depends on photosynthesis for fixed carbon compounds, NAD(P)H, and energy (Turpin et al. 1988, Young and Beardall 2003). Hence, photosynthesis and N assimilation in algae are strongly linked, but the resources required by these processes may not be simultaneously available to intertidal algae.

Studying intertidal organisms is difficult because of the rapidly changing environment, so that the responses of intertidal algae to specific environmental variables have not been well characterized. However, long-term acclimation of macroalgae to particular tidal and light regimes clearly affects nutrient acquisition (Phillips and Hurd 2004), and

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thus the enzymes involved in nutrient assimilation. Examining the activity of enzymes that regulate nutrient assimilation is an effective way to examine nutrient acquisition in macroalgae exposed to different environmental variables, including tidal regime. Measuring the activity of the enzyme nitrate reductase (NR, EC 1.6.6.1) is a good example. NR catalyzes the reduction of nitrate to nitrite, which is usually identified as the rate-limiting step in nitrate assimilation by algae. NR activity has been shown to be potently inhibited by ammonium, a more reduced N form, in unicellular algae (Berges et al. 1995, Vergara et al. 1998) and in a brown macroalga, *Giffordia mitchellae* (Harv.) Hamel (Weidner and Kiefer 1981). Ammonium also suppressed NR protein synthesis in a green macroalga (Balandin and Aparicio 1992) and in a diatom (Vergara et al. 1998). NR activity is strongly correlated with rates of assimilation of nitrate and N incorporation in both phytoplankton (Berges et al. 1995) and macroalga (Davison et al. 1984).

The nature of the variation in NR activity in response to environmental variables, such as light, may give insights into the conditions that particular intertidal macroalgae are experiencing. Regulation of algal NR activity by light has been shown in a range of microalgae (Berges et al. 1995, Ramalho et al. 1995) and macroalgal taxa (Davison and Stewart 1984a,b, Gao et al. 1992, Figueroa 1993, Korb and Gerard 2000). NR synthesis and maintenance of NR activity requires recent exposure to nitrate and is inhibited by ammonium, a more reduced form of inorganic N (Vergara et al. 1998). NR activity is influenced by circadian rhythms in plants (Pilgrim et al. 1993), but although circadian rhythms influence growth in *Laminaria* (Lüning 1994) and photosynthesis in some red macroalgae (Goulard et al. 2004), it is still largely unknown if NR activity in algae is also influenced by circadian rhythms (but see Ramalho et al. 1995).

Comparing variation in NR activity in macroalgae from different regions within the intertidal and subtidal zones provides an ideal means to examine effects of tidal emersion and light exposure, and inorganic N source, on N metabolism. *Fucus* species grow in the intertidal zone of Strangford Lough, Ireland, where they are exposed to the air for several hours during low tide. *Laminaria* species occur in the low intertidal-subtidal margin and are emersed for significant periods only during spring tides. They are covered with up to 3 m of water during high tide, which reduces light availability. These two highly productive brown algal genera experience distinct light and exposure regimes that may influence the regulation of nutrient acquisition and assimilation. In Strangford Lough, inorganic N is mostly available as nitrate, but ammonium is also present; response of N metabolism to these inorganic N sources can also be investigated using NR activity.

To investigate NR activity in these intertidal algae, we employed a modified in vitro assay described by Young et al. (2005) to measure NR activity in *Laminaria digitata* and three *Fucus* species collected from Strangford Lough. In vitro NR activity is strongly correlated with nitrate incorporation rates (Berges et al. 1995), and high frequency sampling shows rapid changes in activity in response to changes in light conditions. Here, we show that NR activity in *L. digitata* displays the patterns of diel variation and sensitivity to light and ammonium typically found in higher plants and most algae. In contrast, we show for the first time that diel variation in NR activity is absent in intertidal *Fucus* species, and that NR activity remains unaffected by additions of ammonium.

MATERIALS AND METHODS

Sampling and experimental incubations. The complete sampling and incubation regime for all experiments is summarized in Table SI (see the supplementary material).

Diel NR activity in L. digitata *under natural irradiance:* Thalli of *L. digitata* were sampled from a boat in Strangford Lough at Portaferry, Northern Ireland (54°23′ N, 5°34′ W), in the middle of the day (1300 h). Collections and incubations to examine diel variation in NR activity were repeated in November, February, April, June, and August. Pieces of thalli were immediately wiped clean, blotted dry, and frozen in liquid N_2 for later nitrate reductase (NR) activity assays. Whole thalli were also transferred within 10 min of collection to outdoor tanks at Queen's University Marine Laboratory at Portaferry, where they were incubated in ambient light conditions and supplied with flowing Strangford Lough seawater at ambient temperature (10°C–13°C), replacing the total tank volume (~250 L) every 15–60 min. Samples of tank inflow and outflow water were collected, and NO_3^- concentrations were measured as 2–8 μmol · L^{-1}. Direct sampling of *L. digitata* from the water close to midnight was not practical, and the tank incubations were designed to mimic the natural environment as closely as possible. During the night following collection (2350 h), samples of *L. digitata* were collected from the tank-incubated thalli. To explore more detailed diel variation, four to eight replicate thalli were collected at the morning low tide (0500–0900 h) and transferred immediately to the outdoor tanks for two light treatments. The same day, initial tissue samples were collected from all thalli, and then half of the thalli were transferred to tanks covered with black plastic sheeting to exclude light for the rest of the experiment, while the other half remained exposed to ambient light conditions. One tissue sample was collected from each thallus (eight thalli per treatment) every 2–4 h over the next 26 h period. *Laminaria digitata* was sampled as described previously (Young et al. 2007). Within a few minutes of all sampling times, tissue samples were thoroughly blotted dry, frozen, and stored in liquid N_2 for later analysis of NR activity.

Circadian variation in NR activity: To examine the possible involvement of circadian rhythms, an additional, longer diel trial over four day-night cycles in April was carried out with four replicate *L. digitata* thalli collected from the Portaferry site (54°23′ N, 5°34′ W) and transferred immediately to tanks supplied with flowing Strangford Lough seawater as described above. The same day, the thalli were incubated in each of three light treatments: ambient light, continuous darkness (as described above), and continuous light in tanks shielded from ambient natural light but receiving 50–150 μmol quanta · m^{-2} · s^{-1} from two lamps (1000 Watt Quartz Halogen, Sylvania

Osram, Langley, UK) suspended above the tank surface. Samples were collected from the thalli as described above.

NR activity in brown macroalgae in laboratory light conditions: Whole thalli of *L. digitata* and *F. vesiculosus* were collected at the Portaferry site described above, transported to the laboratory in Belfast, and placed in 20 L clear plastic tanks with freshly collected, aerated seawater at 12°C–14°C. *Fucus vesiculosus* was collected and incubated in October 2000, and *L. digitata* in November 2001. Thalli were equilibrated in the tanks with natural light periodicity (10:14 light:dark [L:D] cycle) overnight. The next morning, after an initial sampling, the thalli were exposed to either a light regime mimicking the natural periodicity, continuous darkness, or continuous irradiance. The irradiance was 220 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ supplied to one side of the tanks from cool-white fluorescent tubes. Three replicate tanks were used for each treatment, each containing a single thallus. Every 3–4 d, between 1100 and 1300 h, one tissue sample was excised from each thallus (as described for diel experiments), blotted dry, and frozen in liquid N_2 for later NR activity assays. At the beginning of the experiment, and every 3–4 d, the seawater was enriched with $NaNO_3$ (75 μmol $\cdot L^{-1}$) and NaH_2PO_4 (15 μmol $\cdot L^{-1}$), and water samples were also collected to check nutrient concentrations. NO_3^- concentration varied between <0.5 μmol $\cdot L^{-1}$ prior to additions and 80 μmol $\cdot L^{-1}$ immediately after additions, and the NO_2^- concentration in the medium was always <3 μmol $\cdot L^{-1}$. Total carbohydrate levels were measured in *L. digitata* thallus tissue from each sampling date.

Diel NR activity in Fucus *species under natural irradiance:* Samples of *F. serratus, F. vesiculosus,* and *F. spiralis* were collected directly from the shoreline at the Portaferry site at low tide during the day (1400 h) and night (2345 h), blotted dry, and frozen immediately in liquid N_2 for later NR activity measurement. To examine diel activity in more detail, whole thalli of *F. serratus, F. vesiculosus,* and *F. spiralis* were collected from the intertidal region and transferred to the outdoor tanks for diel experiments, which were carried out in the same way as described above for *L. digitata. Fucus* thalli were sampled by removing 30–40 mm long terminal tips (trials showed activity was the highest in tips), sampling uncut tips for successive samplings of the same thallus. Diel incubations were repeated in February, April, June, and November for *Fucus* species.

Effects of ammonium on NR activity: The effect of NH_4^+ enrichment on NR activity was investigated in tank-incubated *F. serratus* and *L. digitata* collected from the Portaferry site and equilibrated overnight in laboratory tanks as described above. For each species, three replicate NH_4^+-enriched tanks and three control tanks were used, each containing a single thallus with 20 L seawater and supplied with light and aeration as described above. The water was enriched twice daily with 50 μmol $\cdot L^{-1}$ NH_4Cl and daily with 15 μmol $\cdot L^{-1}$ NaH_2PO_4 for 4 d; control tanks received only 15 μmol $\cdot L^{-1}$ NaH_2PO_4. Thalli were subsampled initially and then after 4 d; samples were blotted dry and frozen in liquid N_2 for later NR activity assays.

Statistical analysis: Preliminary samples were used to test the variance in NR activity between and within thalli. An *F*-test showed that the variability of NR activity in six tips from each thallus was higher than, but not significantly different from, the variability of NR activity in tips from six different individuals ($P > 0.05$). From this finding, we concluded that although the same thalli were sampled repeatedly, it was not necessary to treat this procedure as a repeated measures sampling for statistical purposes. To make statistical comparisons of NR activity over diel cycles, we pooled values from all samples collected during the light period and compared these with pooled values from all samples collected during the dark period, using one-way or two-way analysis of variance (ANOVA; Sigmastat v. 3.1, Systat Software Inc., Chicago, IL, USA). Sampling during twilight was avoided.

Analytical methods. Inorganic N determinations: NO_3^- was analyzed by Cd-column reduction followed by spectrophotometric measurement of NO_2^-, and NH_4^+ was estimated by the phenol-hypochlorite method, both according to Parsons et al. (1984).

Total carbohydrate levels: Total carbon–hydrogen–oxygen (CHO) levels were measured in *L. digitata* thallus tissue using 0.15% (w/v) anthrone reagent (Sigma Inc., St. Louis, MO, USA) according to Parsons et al. (1984) using D-glucose (Sigma Inc.) as a standard.

NR activity: Activity of NR (EC 1.6.6.1) was estimated using the assay method described by Young et al. (2005). Frozen thallus samples were ground to a powder in liquid N_2 and extracted in 200 mmol $\cdot L^{-1}$ potassium phosphate buffer pH 7.9 with 5 mmol $\cdot L^{-1}$ Na_2EDTA, 0.3% (w/v) insoluble polyvinyl pyrollidone, 2 mmol $\cdot L^{-1}$ DL-dithiothreitol, 3% (w/v) BSA (Fraction V), and 1% (v/v) Triton X-100 (all chemicals from Sigma Inc.). The assay mixture contained 200 mmol $\cdot L^{-1}$ sodium phosphate buffer pH 7.9 with 200 μmol $\cdot L^{-1}$ NADH (β form), 20 μmol $\cdot L^{-1}$ flavin adenine dinucleotide, 10 mmol $\cdot L^{-1}$ KNO_3 (all chemicals from Sigma Inc.) and 20% volume as the algal extract. For all samples, the assay was incubated at 13°C and terminated by addition of 1 M zinc acetate. NO_2^- concentration was measured spectrophotometrically in clarified supernatants, as described above, and NR activity was estimated by linear regression of increasing NO_2^- concentration over time.

RESULTS

NR activity in *L. digitata* collected during ambient light conditions in February 2001 was 5-fold higher in thalli sampled in the day than in the night ($P < 0.001$; Fig. 1). Day-night differences in NR activity in *L. digitata* could also be seen in the more detailed diel patterns shown in Figure 2. In thalli exposed to ambient light conditions during February, April, and November 2001, there was a significantly lower NR activity during the dark period than during the light (one-way ANOVA, $P < 0.002$). Transfer to continuous darkness, after the initial

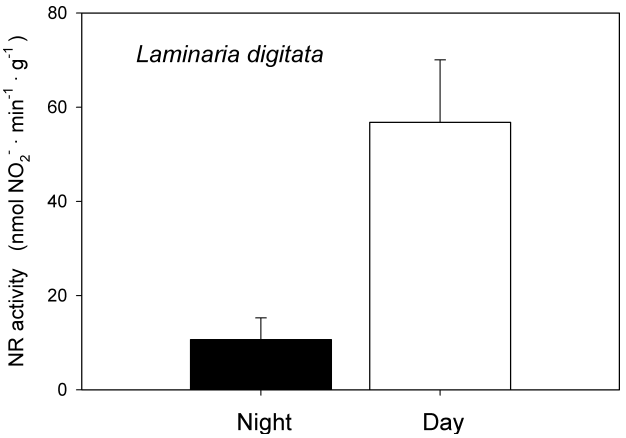

FIG. 1. Day-night differences in nitrate reductase (NR) activity in *Laminaria digitata* sampled from a boat in the middle of the day (1300 h) and at night (2350 h) from outdoor tanks exposed only to natural light conditions, in February 2001. Bars are mean + SD; $n = 3$. The two treatments were significantly different ($P < 0.001$).

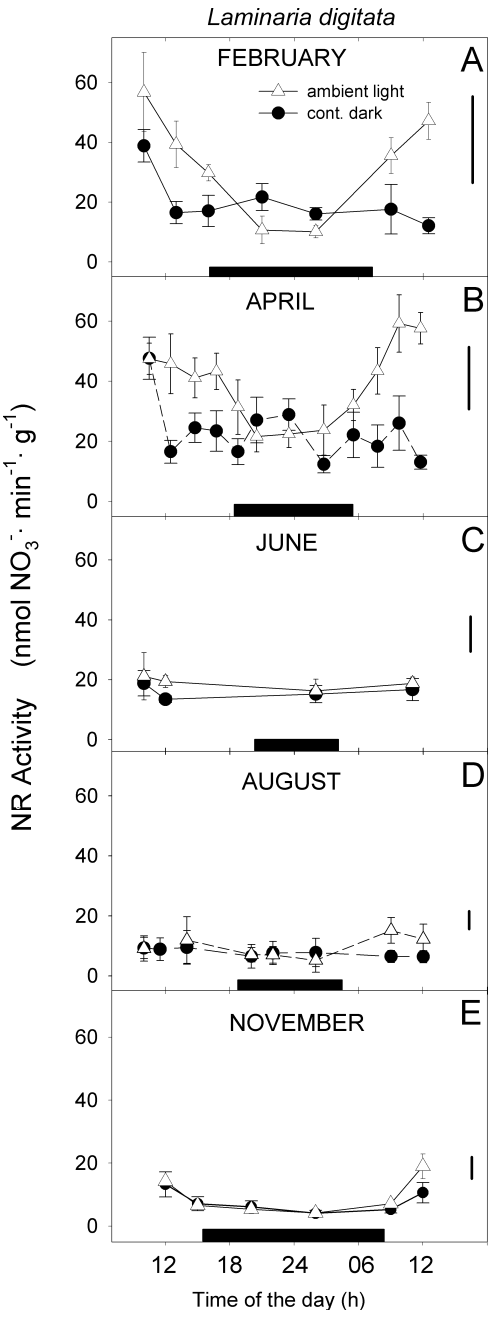

FIG. 2. Diel variation in nitrate reductase (NR) activity measured in *Laminaria digitata* incubated in darkness (solid symbols) and in ambient light (open symbols) during five separate months. The first point represents NR activity in thalli acclimated to ambient light, before transfer to darkness. Points are mean NR activity measured in samples from four to eight different thalli; bars are SD. Vertical bars on each plot represent the least significant difference at $P = 0.05$. The dark period for each time of sampling is indicated by a black bar on the *x*-axis. Light versus dark treatments were significantly different in February and April ($P < 0.001$), and time of day was significant in ambient light treatment in February ($P < 0.002$), April ($P < 0.045$), and November ($P < 0.002$).

sampling, decreased NR activity to a relatively constant and low value (Fig. 2). In November, the diel variation was significant (one-way ANOVA, $P < 0.002$) but less marked than in April; and in June and August, activities were very low, and there was no significant diel variation in NR activity (two-way ANOVA, $P > 0.36$). There were large differences in NR activities seasonally, with the lowest activities during August and November, and the highest in February and April (Fig. 2). In June, August, and November, the decrease in NR activity after transfer to darkness was not significant ($P > 0.47$), but there was a significant diel variation observed in the ambient light treatment in November (two-way ANOVA, $P < 0.002$).

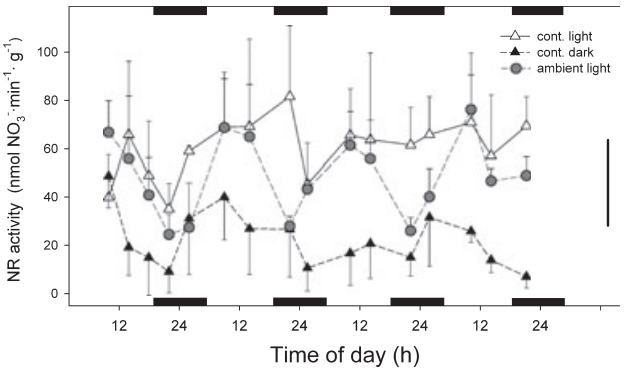

FIG. 3. Extended analysis of diel variation of nitrate reductase (NR) activity in *Laminaria digitata* exposed to ambient light (shaded circles, ~14:10 L:D), continuous darkness (solid triangles), and continuous light (open triangles) in outdoor tanks during April 2002. Symbols are means ± SD ($n = 4$); vertical bar represents least significant difference at $P = 0.05$. Dark periods for the ambient treatment are indicated by black bars on the *x*-axis. The first point represents NR activity in thalli acclimated to ambient light, before transfer to the light treatments. Diel variation in NR activity was significant under ambient light exposure ($P < 0.001$), but in continuous light and continuous dark, NR activity did not vary significantly with time of day ($P > 0.34$ and $P > 0.16$ for light and dark, respectively).

To explore whether there was a circadian rhythm involved in the diel variation in NR activity in *L. digitata*, NR activity was observed in longer, 4 d incubations (Fig. 3). Diel variation of NR activity in *L. digitata* exposed to ambient light was consistent over the 4 d experimental incubation, showing significantly higher NR activity during the light period than at night (one-way ANOVA, $P < 0.001$). There was a significantly higher NR activity in samples from the continuous light treatment than in those from the continuous dark treatment (Fig. 3; one-way ANOVA, $P < 0.001$). However, both continuous light and dark treatments showed no significant difference in NR activity between samples collected during the natural ambient daytime and ambient nighttime (one-way ANOVA, $P > 0.5$ for continuous light, $P > 0.3$ for dark). There were no significant differences in NR activity with time of day or over the 4 d for the continuous light treatment (two-way ANOVA, $P > 0.96$ for time, $P > 0.14$ for day) or for the continuous dark treatment (two-way ANOVA, $P > 0.5$ for time, $P > 0.2$ for day).

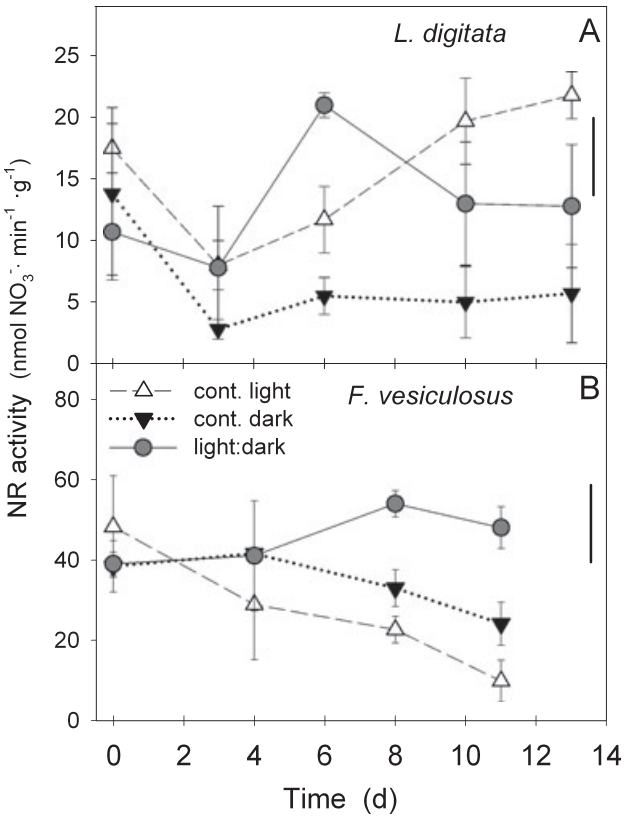

FIG. 4. Effect of light regime on nitrate reductase (NR) activity in laboratory culture of *L. digitata* (A) and *F. vesiculosus* (B). Symbols are mean NR activity ± SD for samples taken from three replicate thalli incubated in continuous light (open triangles), continuous darkness (solid triangles), or natural light periodicity (10:14 L:D; shaded circles). Vertical bars on each plot represent least significant difference at $P = 0.05$. The first point indicates the initial sampling prior to transfer to the treatment light regime.

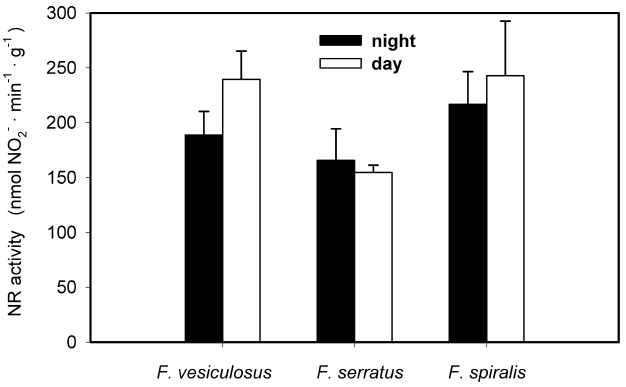

FIG. 5. Day-night differences in nitrate reductase (NR) activity in *Fucus* spp. *F. vesiculosus*, *F. serratus*, and *F. spiralis* sampled directly from the shore at low tide during the day (1400 h) or at night (2345 h) in November 2000. Bars are mean + SD; $n = 4$. There was no significant effect of time of day in each *Fucus* species ($P = 0.179$).

The effects of continuous light and dark treatments on NR activity were examined in *L. digitata* and *F. vesiculosus* in laboratory cultures. In *L. digitata,* transfer to continuous darkness in laboratory cultures resulted in a lower NR activity after 3 d, and a constant low NR activity was observed over the next 10 d (Fig. 4A). Continuous light treatment increased NR activity in *L. digitata* up to day 13 (one-way ANOVA, $P < 0.005$). In control *L. digitata* thalli, which were exposed to an L:D cycle similar to the natural periodicity (10:14), there was no significant change in NR activity over the experiment (one-way ANOVA, $P > 0.05$; Fig. 4). There were no significant changes in carbohydrate content of *L. digitata* over time or with light treatment over the 13 d experiment (two-way ANOVA, $P > 0.1$; data not shown). In contrast to the responses of *L. digitata*, there was no significant change in NR activity in *F. vesiculosus* in continuous darkness until day 11 when, despite the extended light deprivation, NR activity in thalli declined to just over 50% of the initial activity (Fig. 4B). Also in contrast to *L. digitata*, exposure to continuous light resulted in a significant decline in NR activity in *F. vesiculosus* over 11 d (one-way ANOVA, $P < 0.01$). There was no significant change in NR activity in *F. vesiculosus* thalli exposed to 11 d of 10:14 L:D cycle (one-way ANOVA, $P > 0.05$; Fig. 4).

Differences between the responses of *F. vesiculosus* and *L. digitata* to light regimes in laboratory cultures were explored in the field and in additional *Fucus* species. There were no differences between the NR activity in *F. vesiculosus, F. serratus,* and *F. spiralis* thalli sampled directly from the shore at low tide, near the middle of the day and in the middle of the night, in November 2000 ($P > 0.3$; Fig. 5). These results suggested a lack of diel variation in NR activity in these species, which was confirmed in a more detailed sampling from outdoor-tank experiments (Fig. 6). There was no significant difference between NR activity from thalli transferred to continuous darkness and those in ambient light (one-way ANOVA, $P > 0.5$), and there was no diel variation in NR activity in thalli of *F. vesiculosus*, *F. serratus* (Fig. 6), and *F. spiralis* (data not shown) exposed to ambient light (two-way ANOVA, $P > 0.08$). This lack of diel variation and independence of light in *Fucus* species was consistent in February, April, June, and November, although there were seasonal differences in the magnitude of NR activity. Like *L. digitata* (Fig. 2), higher NR activities were observed in February and April, with the lowest activities in June. NR activities in *F. vesiculosus* in November were similar to those in February (Fig. 6). NR activity in *Fucus* species was always higher than in *L. digitata,* both in field samples (Fig. 1 cf. Fig. 5; Fig. 2 cf. Fig. 6) and in laboratory experiments (Fig. 4).

In addition to these differences between NR activity in *L. digitata* and in *Fucus* species in response to light and time of day, NR activity in *L. digitata* and *F. serratus* also showed distinct responses to elevated

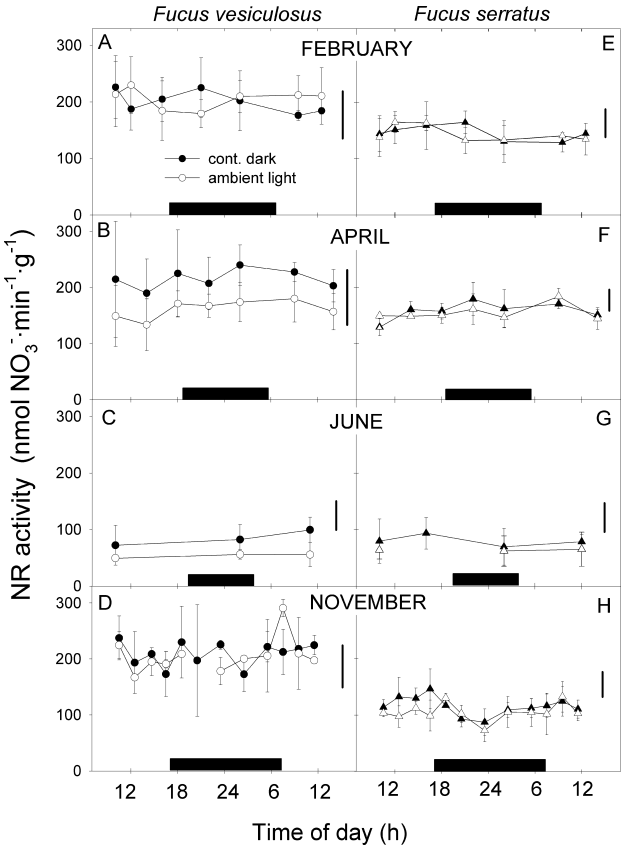

FIG. 6. Diel variation nitrate reductase (NR) activity in *Fucus* incubated in darkness (solid symbols) and in ambient light (open symbols) during four separate months. (A–D) *F. vesiculosus*. (E–H) *F. serratus* ($n = 4$–5). Other details as for Fig. 2. Light and dark treatments were not significantly different in *F. serratus* and *F. vesiculosus* ($P > 0.08$).

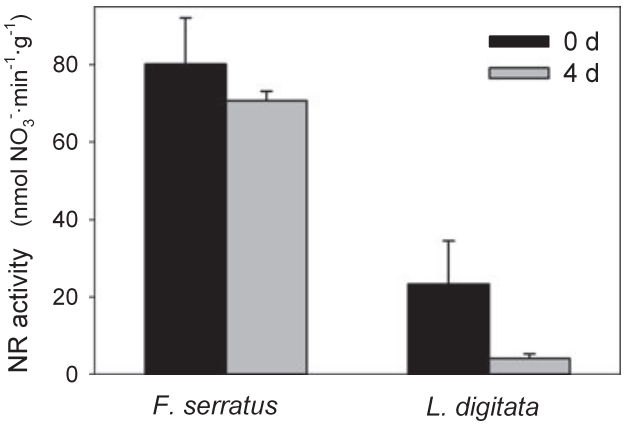

FIG. 7. Effect of exposure to twice daily pulses of elevated NH_4^+ (50 μmol · L^{-1}) on nitrate reductase (NR) activity in *F. serratus* and *L. digitata* incubated in laboratory tanks for 4 d. After 4 d, NR activity was significantly suppressed in *L. digitata* ($P < 0.003$), but not in *F. serratus*. Bars are mean + SD; $n = 3$.

ammonium concentration (Fig. 7). When exposed to pulses of 50 μmol · L^{-1} NH_4^+ given twice daily over 4 d, NR activity in *L. digitata* declined by 80% (one-way ANOVA, $P < 0.003$) but did not decline significantly in *F. serratus* (one-way ANOVA, $P > 0.4$; Fig. 7). In both species, there was no significant change in NR activity in thalli incubated in natural seawater without NH_4^+ enrichment (one-way ANOVA, $P < 0.1$; data not shown).

DISCUSSION

NR activity was measurable throughout the year in *L. digitata* and in the three *Fucus* species collected from Strangford Lough. This finding confirms the utility of the in vitro assay (Hurd et al. 1995), modified by Young et al. (2005) to improve extraction efficiency, despite earlier reports of poor or no activity from an in vitro NR assay method (Corzo and Niell 1991). The in vitro approach was useful because rapid freezing and storing for later analysis enabled well-replicated, frequent sampling over time, which would not have been possible using an in situ method that requires assays to be performed immediately (Corzo and Niell 1991, Lartigue and Sherman 2002). Furthermore, the in vitro approach avoided the need for up to 30 min dark incubation for the in situ method (Corzo and Niell 1991), which, in this study, would have resulted in a significant suppression of NR activity in *L. digitata* during the assay period.

Diel variation of NR activity in L. digitata. The pronounced diel pattern of NR activity observed in *L. digitata* incubated in outdoor tanks during November, February, and April showed high NR activity near the middle of the day and suppression during darkness. It was also consistent with previously reported observations in *L. digitata* (Davison and Stewart 1984a,b) and other macroalgae (Gao et al. 1992, Lartigue and Sherman 2002), and similar to patterns found in phytoplankton (Berges et al. 1995). In diatoms, an increase in NR activity toward the end of the dark period has been noted (Berges et al. 1995, Vergara et al. 1998), but this has not been observed in macroalgae or higher plants. However, a decline in NR activity was observed in *L. digitata* within 2 h following transfer from light to dark, and our preliminary experiments suggest that this decline can occur more rapidly, within 30 min. Although diel regulation of algal NR activity can involve protein synthesis (Ramalho et al. 1995, Vergara et al. 1998), the rapid suppression observed in *L. digitata* suggests involvement of a posttranslational regulation of NR activity in this alga, which would allow very rapid changes in activity in response to light, for example, as the thalli become heavily shaded at high tide or at sunset.

The marked seasonal differences in NR activity may be related algal responses to temperature, irradiance, and day length, which have been discussed by Young et al. (2007), and may involve regulation of NR activity in response to a circannual rhythm (Gómez and Lüning 2001). In the summer, when

the algae are exposed to more intense sunlight for nearly 18 h a day, peak NR activities were lower than in winter and spring, and there was no significant diel NR activity variation or suppression of NR activity during the short dark period. In summer, nitrate assimilation will be limited by light for a much shorter period over each diel cycle, and *L. digitata* may be able to assimilate sufficient nitrate to support growth with less NR activity, but over a longer period each day. Conversely, in winter, NR activity needs to be higher to compensate for low irradiance and short days, which energetically limit N assimilation and growth. When nitrate is available, phytoplankton show a marked diel periodicity in N uptake, but this pattern breaks down when N becomes limiting (Cochlan et al. 1991). Assuming nitrate uptake and NR activity are closely linked, a similar pattern may explain the lack of a diel variation in NR activity in *L. digitata* over the summer and autumn months, when water-column nitrate availability is likely to be the lowest (DARDNI database 2006, Young et al. 2007). A previous report of diel NR activity in *L. digitata* (Davison and Stewart 1984a,b) showed only data from May to June. How strongly a diel signal is observed in NR activity in field-grown algae may depend on the time of year the algae are collected.

Despite marked diel variation and obvious light regulation of NR activity in *L. digitata,* a low level of NR activity was maintained in the dark, even after 13 d (Fig. 4). This apparently light-independent activity may be due to a constitutive NR enzyme activity that is insensitive to environmental regulation. Furthermore, in the outdoor-tank experiment during June and August, when NR activity was at a seasonal low, the light-induced activity was not significantly higher than the dark activity. This background level may also represent a constitutively expressed, light-insensitive NR activity. In higher plants, both inducible and constitutive forms of NR have been found, and although the inducible NR is regulated by environmental factors (light, nitrate), constitutive expression of another form maintains a constant activity rate (Yang and Midmore 2005). A similar situation may account for the maintenance of low levels in darkness and in elevated NH_4^+ conditions in *L. digitata*, although whether the inducible and the apparently constitutive activities are due to different NR genes is yet to be investigated.

The diel pattern of NR activity in *L. digitata* in November, February, and April was similar to that reported in phytoplankton, subtidal algae, and higher plant species, none of which experience the tidal cycles that affect intertidal *Laminaria* species. This suggests that light is also the dominant factor involved in diel regulation of NR activity in *L. digitata.* Following prolonged dark incubation, when polar *Laminaria solidungula* J. Agardh thalli were transferred back to the light, NR activity increased 20-fold (Korb and Gerard 2000). The effect of light on NR activity in *L. digitata* may be direct, involving energy, or mediated through photosynthesis and its products. There is also strong evidence from higher plants and other algae for a connection between C metabolism and other regulation of N assimilation (Turpin et al. 1988, Figueroa 1993, Young and Beardall 2003), and for direct effects of photosynthesis on NR regulation (Vergara et al. 1998, Yang and Midmore 2005). Vergara et al. (1998) also linked diel variation in NR in the diatom *Thalassiosira weissflogii* (Grunow) G. A. Fryxell et Hasle with daily oscillations in internal C:N ratio, suggesting that the predawn rise in NR activity they observed was a response to declining cellular C content (low C:N) at the end of the dark period (Vergara et al. 1998). A similar anticipation of the light period in NR activity was not observed for *L. digitata*, and NR activity increased only after the transfer to the light period but increased steadily during the morning and declined in the afternoon (Fig. 2, A and B). However, large daily fluctuations in cellular C content, which may occur in single-celled algae, are less likely in *Laminaria*, which can store significant quantities of organic C in the large thalli (Henley and Dunton 1997). Even after light deprivation for 13 d, there was no significant depletion of internal carbohydrate levels in *L. digitata.* C:N ratios in *L. digitata* do fluctuate seasonally (Young et al. 2007).

Diel oscillation in Laminaria *NR—circadian rhythm?* The diel monitoring of NR activity over 4 d in *L. digitata* exposed to ambient light, continuous light, and dark conditions showed that despite the strong diel variation in NR activity, this pattern did not persist in continuous light or dark conditions. This finding is evidence that the periodicity of ambient light, rather than an endogenous circadian rhythm, was regulating diel NR activity in *L. digitata.* While endogenous rhythms control many metabolic and physiological events in algae (Lüning 1994, Suzuki and Johnson 2001), the regulation of NR by an endogenous rhythm in macroalgae, independent of light-dark exposure, is yet to be convincingly demonstrated. Several 24 h cycles in continuous light conditions must be examined in replicate thalli (Suzuki and Johnson 2001), such as those used to examine circadian oscillation of NR mRNA in *Arabidopsis* (Pilgrim et al. 1993), to provide robust evidence for an endogenous circadian rhythm. The current study provides evidence that diel NR variation in *L. digitata* is not regulated by an endogenous rhythm but is dependent on light, or some light-regulated metabolite. Even in higher plants, for which robust evidence for circadian rhythm involvement in NR activity is available (see Pilgrim et al. 1993), recent analysis suggests that circadian oscillation in NR is probably related to substrate feedback and not an endogenous ''clock'' (Yang and Midmore 2005). More detailed studies on a range of macroalgae, including monitoring NR over several days, are needed to show whether NR is regulated

by a light-independent circadian rhythm in other macroalgal species.

Novel patterns of NR activity in Fucus *species.* NR activity in all three species of *Fucus* examined showed no evidence for diel variation, either when collected directly from the intertidal zone or incubated in tanks exposed to ambient light conditions, or for rapid suppression of NR activity following transfer of thalli to darkness. This lack of light sensitivity and diel pattern is novel and contrasts with the strong light regulation of NR previously reported for numerous plants and other algae.

Another difference between NR activity in *Fucus* and other algal species examined to date is the response to ammonium enrichment. Ammonium has been observed to inhibit NR activity, and NR protein synthesis, in several algae (Balandin and Aparicio 1992, Berges et al. 1995, Vergara et al. 1998). However, although elevated ammonium concentrations reduced NR activity in *L. digitata*, NR activity in *F. serratus* was not significantly affected by 4 d of exposure to high ammonium concentration. The apparent lack of light and ammonium regulation of NR activity in *Fucus* diverges from the strong paradigm of ammonium and light regulation of NR in other algae and higher plants. These differences in regulation may be due to differently regulated NR isozymes in *Fucus* and *Laminaria*. Different isozymes may also account for both apparently constitutive and light-regulated NR activity in *L. digitata*.

Although species of both *Laminaria* and *Fucus* inhabit temperate intertidal zones in the North Atlantic, *F. serratus, F. vesiculosus,* and *F. spiralis* all occur higher in the intertidal zone and experience more prolonged exposure to the air than the *Laminaria* species. There would be little advantage for intertidal *Fucus* to use light-dark cues to synchronize N metabolism to exploit periods of immersion, which are determined by tidal cycles and are thus offset from photoperiod. The maintenance of high NR activity throughout the day and night may be a response to this disjunction between light availability and periods of emersion. Seaweeds growing higher in the intertidal zone also show higher uptake rates for inorganic nutrients than subtidal species, possibly because thalli have access to nutrients only during the briefer periods of immersion (Phillips and Hurd 2004). The higher intertidal habitat thus imposes daily temporal limitation for N. In phytoplankton experiencing concentration limitation for N, light dependence of N uptake is lost (Cochlan et al. 1991), and nitrate assimilating capacity may follow the trend in N uptake. Murthy et al. (1986) reported higher NR activity within algal species growing higher in the intertidal zone, and NR activities measured in the three *Fucus* species and in other mid- and upper-intertidal fucoid algae, *Ascophyllum nodosom* (L.) Le Jol. and *Pelvetia canaliculata* (L.) Decne. et Thur., were also higher than in *L. digitata* (Young et al. 2005). In *L. digitata*, however, which is immersed for the majority of the time, nitrate uptake (and assimilation) can occur almost throughout the full diel cycle, provided that energy (light) is available. Hence, there may be an adaptive advantage to maintain predominant light regulation of NR activity in *Laminaria*. This adaptation may contribute to lower mass-specific NR activity levels and to the strongly light-regulated NR activity in *Laminaria*. Further studies to evaluate a habitat-based explanation for the differences in regulation of NR between the two groups of brown algae are warranted. The question remains whether the different patterns of NR expression between *Fucus* spp. and *L. digitata* are due to adaptation to the different environments they inhabit or to a fundamental evolutionary origin. Phylogenetic distance may contribute to divergence of NR genes between *Fucus* and *Laminaria*. Nothing is known about NR genes in macroalgae, and microalgal NR genes have been examined only recently. These show sequence homology with higher plant NR genes (Allen et al. 2005), and the most sequence divergence is in the N-terminal region, which is also responsible for light regulation in plant NR genes (Nussaume et al. 1995). Analysis of this region of brown algal NR genes could provide insights into the different light and ammonium sensitivity of NR activity in *Fucus* and *Laminaria*.

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Supplementary Material

The following supplementary material is available for this article.

Table S1. Outline of seaweed sampling regime for each experiment. All samples were collected from Portaferry site (PF) (54°23′ N, 5°34′ W). Abbreviations: Ld, *Laminaria digitata*; Fv, *Fucus vesiculosus*; Fs, *F. serratus*; Fsp, *Fucus spiralis*; FT-PF, flow-through tanks at Portaferry; ALT, aerated 20 L laboratory tanks; AM, between 0500 and 0900 h; cont., continuous (light treatment). Time collected refers to time when thalli were collected from the natural environment, and tissue sampling refers to when pieces of the thalli were sampled and frozen for later analysis. Details are explained in Materials and Methods section.

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