

# Physiological responses of intertidal marine brown algae to nitrogen deprivation and resupply of nitrate and ammonium

Erica B. Young<sup>a,b,\*</sup>, John A. Berges<sup>a,b</sup> and Matthew J. Dring<sup>b</sup>

<sup>a</sup>Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA

<sup>b</sup>School of Biology and Biochemistry, Queen's University of Belfast, Belfast, Northern Ireland, BT7 1NN, UK

## Correspondence

\*Corresponding author,  
e-mail: ebyoung@uwm.edu

Received 28 October 2008; revised 9  
December 2008

doi: 10.1111/j.1399-3054.2008.01199.x

Intertidal macroalgae *Fucus* and *Laminaria* experience seasonally fluctuating inorganic N supply. This study examined the effects of long-term N deprivation, recovery following N resupply, and effects of elevated ammonium and nitrate exposure on N acquisition in intertidal algae using manipulations of N supply in tank culture. Over 15 weeks of N deprivation, internal N and nitrate reductase activity (NRA) declined, but maximum quantum yield of PSII was unaffected in *Fucus serratus* and *Fucus vesiculosus*. Low NRA was maintained despite no external nitrate availability and depletion of internal pools, suggesting a constitutive NRA, insensitive to N supply. Nitrate resupplied to N-starved thalli was rapidly taken up and internal nitrate pools and NRA increased. Exposure to elevated (50  $\mu\text{M}$ ) nitrate over 4 days stimulated nitrate uptake and NRA in *Laminaria digitata* and *F. serratus*. Exposure to elevated ammonium suppressed NRA in *L. digitata* but not in *F. serratus*. This novel insensitivity of NRA to ammonium in *Fucus* contrasts with regulation of NRA in other algae and higher plants. Ammonium suppression of NRA in *L. digitata* was not via inhibition of nitrate uptake and was independent of nitrate availability. *L. digitata* showed a higher capacity for internal nitrate storage when exposed to elevated ambient nitrate, but NRA was lower than in *Fucus*. All species maintained nitrate assimilation capacity in excess of nitrate uptake capacity. N uptake and storage strategies of these intertidal macroalgae are adaptive to life in fluctuating N supply, and distinct regulation of N metabolism in *Fucus* vs *Laminaria* may relate to position in the intertidal zone.

## Introduction

Brown macroalgae are dominant in temperate marine coastal environments and contribute the majority of the primary production in these environments, where productivity is typically limited by supply of inorganic nitrogen (Dugdale 1967). Strongly, seasonal oscillations and tidal fluctuations in water level influence light and nutrient supply to brown macroalgae, and their capacity

to take up and assimilate nitrogen is critical to energy and nutrient cycling in nearshore environments.

In the temperate Strangford Lough, Northern Ireland, the nearshore macroalgal community is dominated by species of *Laminaria* and *Fucus*. Nitrate is the major form of dissolved inorganic N available in this environment. Intertidal macroalgae experience strong seasonal fluctuations in nitrate supply (Young et al. 2007a), and tidal

**Abbreviations** – DW, dry weight;  $F_v/F_m$ , maximum quantum yield of PSII; FW, fresh weight; NR, nitrate reductase; NRA, nitrate reductase activity.

emersion isolates the algae from supply of dissolved nutrients. Nitrate assimilation capacity varies several fold seasonally in these brown algae in response to nitrate availability; in winter, nitrate concentrations are not limiting, but in summer, nitrate is significantly depleted (Young et al. 2007a). The growth of temperate brown algae can be limited by inorganic N supply, particularly during the summer months (Chapman and Craigie 1977, Naldi and Viaroli 2002, Pedersen and Borum 1996).

Supply of nitrogen is important for annual primary production and nutrient cycling within the nearshore region, but how these algae acclimate to summer N deprivation is not well understood. In contrast, N starvation kinetics and the implications of N deprivation for photosynthesis are well understood in some unicellular algal species (Geider et al. 1993, Young and Beardall 2003). Macroalgae can store more internal N relative to their daily needs than unicellular algae (Dortch et al. 1984, Henley and Dunton 1995, Korb and Gerard 2000, Pedersen and Borum 1996, Poeschel and Korb 2001, Young et al. 2007a), so one might expect that photosynthetic capacity will not be compromised as rapidly by N deprivation. Macroalgae can take up nutrients rapidly (Harrison et al. 1986, Pedersen 1994), and after prolonged N starvation, non-constitutive nitrate uptake and assimilation capacity might be expected to be activated rapidly to exploit sudden increases in nutrient availability. However, the extent to which brown macroalgae can be starved of N and the physiological responses of N metabolism and photosynthesis to N deprivation and resupply of N have not been studied in detail.

Intertidal algae are supplied with a range of inorganic N sources, and metabolism of inorganic N by algae is known to be regulated by nitrate and ammonium. For many algae, ammonium is the preferred inorganic N form and presence of ammonium can suppress the uptake and assimilation of less reduced forms of N, mainly nitrate, in phytoplankton (Dortch 1990) and macroalgae (DeBoer 1981, Haines and Wheeler 1978, Harrison et al. 1986). Elevated ammonium can also result in a decline in the activity of the primary nitrate-assimilating enzyme, nitrate reductase (NR) in microalgae (Berges et al. 1995, Vergara et al. 1998) and in a brown macroalga, *Giffordia mitchellae* (Weidner and Kiefer 1981). The regulation of inorganic N uptake and assimilation in intertidal seaweeds in response to availability of different inorganic N forms has been investigated and compared in relatively few species.

To expand our understanding of how nitrogen deprivation and supply of different inorganic N sources affect N metabolism in intertidal brown algae, we examined intertidal algae under manipulated tank cultivation conditions to examine three key questions:

(1) What is the effect of long-term N deprivation on N metabolism and photosynthetic capacity in intertidal brown algae?

(2) When resupplied with a source of nitrogen, how rapidly can N-deprived thalli take up and re-establish N assimilation capacity?

(3) What are the effects of exposure to ammonium and nitrate on nitrate uptake and assimilation by intertidal brown algae?

To examine the role of position in the intertidal zone on macroalgal physiology, nitrogen metabolism in lower intertidal–subtidal *Laminaria digitata* (Huds.) Lamour was compared with higher intertidal *Fucus* species.

## Materials and methods

### Effects of tank storage, N deprivation and N resupply

To examine the effect of tank storage on N metabolism, whole thalli of *Fucus serratus* L. and *Fucus vesiculosus* (L.) Lamour were collected in March 2001 from the intertidal region of Strangford Lough at Portaferry (54°23'N, 5°34'W). Thalli were either transferred to outdoor tanks adjacent to the field collection site, with flow-through seawater as described previously (Young et al. 2007b), or transported to the laboratory and incubated in 20-l tanks with freshly collected, filtered, aerated seawater at 11°C. Laboratory tanks were exposed to a light regime similar to the ambient at the time of collection (10:14 L:D cycle) at 220  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  supplied to one side of the tanks from GROW-lux fluorescent light tubes (Osram, Langley, Berkshire, UK). The seawater medium in these tanks was changed on days 5, 7, 20 and 27, and at each change, 75  $\mu\text{M}$   $\text{NaNO}_3$  and 15  $\mu\text{M}$   $\text{NaH}_2\text{PO}_4$  were added to the water. Background concentration of nutrients in freshly collected seawater was 2–8, 0.5–3 and <0.5  $\mu\text{M}$  for nitrate, ammonium and nitrite, respectively (Young et al. 2007a), and dissolved organic N is typically 10–75  $\mu\text{M}$  (G. Savidge, J. Berges, M. Dring, K. Sommerville, unpublished data). Tips of thalli in outdoor and laboratory tanks were excised, blotted dry and frozen in liquid  $\text{N}_2$  for later analysis of nitrate reductase activity (NRA) (Young et al. 2005).

To examine the effect of N deprivation on NRA, whole thalli were also collected in early March 2001 and incubated in laboratory tanks for 15 weeks (until mid June) in freshly collected seawater without any nitrate additions, but with 15  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$  added on days 0, 7 and 27. Thereafter, 15  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$  and 1  $\text{ml l}^{-1}$  enriched seawater, artificial water (ESAW) micronutrients, including vitamins, iron and other trace metals (Harrison et al. 1980),

were added weekly. The initial mass was approximately 120 g for *F. vesiculosus* and approximately 200 g for *F. serratus*. From this N-deprivation treatment, thallus samples were collected at intervals, blotted dry and stored in liquid N<sub>2</sub> until analysis of NRA, extraction of internal nitrate and ammonium pools and analysis of C and N content (Young et al. 2005, 2007a).

Maximum quantum yield of PSII ( $F_v/F_m$ ) was also measured at the beginning of the N-depletion experiment and after 15 weeks using a pulse amplitude modulated fluorometer (PAM-2000; Heinz Walz GmbH, Effeltrich, Germany). Samples were dark acclimated for 20–30 min before  $F_v/F_m$  was measured on eight tips of each thallus with the fibre-optic probe placed against the thallus surface.

To examine the effects of N resupply, after 15 weeks of N deprivation, 100  $\mu\text{M}$  NaNO<sub>3</sub> was added to the seawater. 100  $\mu\text{M}$  may not have saturated uptake mechanisms, but as thalli naturally experience a maximum nitrate concentration approximately <20  $\mu\text{M}$  (Young et al. 2007a), the experiment estimated maximum nitrate uptake capacity under field conditions. Freshly collected thalli were also supplied with 100  $\mu\text{M}$  nitrate and monitored to compare with the N-deprived thalli. Samples were collected from all thalli over the following week to monitor NRA, internal thallus nitrate content and total C and N thallus content. Although *L. digitata* was successfully grown in outdoor tanks (Young et al. 2007b, Gomez and Lüning 2001), a laboratory N deprivation experiment was set up with *L. digitata* alongside that for *Fucus*, but the *L. digitata* thalli fragmented and disintegrated within 4 weeks so that experiment was terminated.

### Effects of inorganic N sources on N metabolism

The effects of elevated inorganic N availability and different N sources on N metabolism in *L. digitata* and *F. serratus* were assessed by incubation in laboratory tanks. Both species were collected from the intertidal region of Strangford Lough at Portaferry in July 2001 and transported to the laboratory. Smaller but whole thalli were selected, any epiphytes were removed and three intact thalli per 20-l tank were acclimated with freshly collected, filtered seawater at 13°C, with aeration and irradiance as above. The initial total thallus mass for each tank was approximately 180 g for *F. serratus* and approximately 125 g for *L. digitata*. After 2–3 days of acclimation, four treatments were established with the following additional nutrients added to the seawater: (1) elevated ammonium – 50  $\mu\text{M}$  NH<sub>4</sub>Cl; (2) elevated nitrate – 50  $\mu\text{M}$  NaNO<sub>3</sub>; (3) elevated ammonium plus nitrate – 50  $\mu\text{M}$  each of NH<sub>4</sub>Cl and NaNO<sub>3</sub>; and (4) control – no N additions. Nutrient additions were made every 12 h to the tanks, except in tank 3, where after 2 days, NaNO<sub>3</sub> and

NH<sub>4</sub>Cl additions were made only every 24 h to compensate for higher total N additions to this tank. NaH<sub>2</sub>PO<sub>4</sub> (16  $\mu\text{M}$ ) was added to all tanks at the start of the experiment and again after 48 h. At each nutrient addition time, the water was sampled before and after nutrient addition for analysis of nitrate and ammonium concentration, as described below. Water samples for nitrate analysis were frozen immediately, and samples for ammonium were analysed the same day or stored overnight in darkness at 4°C prior to analysis. During the first 24 h, additional tissue and water samples were collected at 4, 8 and 24 h to assess short-term effects of elevated N on NRA in thalli and to measure rate of N uptake by the thalli using the initial thallus mass for calculations. For *F. serratus*, thallus tips were sampled, while *L. digitata* were sampled by cutting discs out of the thalli, avoiding the meristematic region and the oldest tissue (Young et al. 2007b). Samples were blotted dry and frozen in liquid N<sub>2</sub>. Tissue samples were also excised at the beginning, after 24 h and at the end of the experiment for extraction and measurement of internal nitrate and ammonium concentration. Samples were thoroughly blotted dry and frozen at –20°C. Total masses of the thalli were measured in each tank at the beginning and at the end of the experiment, and masses of samples removed during the experiment were also recorded.

Following the 4 days of incubation in the N treatments, nitrate uptake rate was examined in the thalli. Each thallus was washed in clean seawater without nutrient additions and transferred to a flask with 2-l clean, fresh seawater at 11°C with vigorous aeration to maintain complete mixing and illuminated with 140  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  light from above. Following acclimation for 1 h, 100  $\mu\text{M}$  NaNO<sub>3</sub> was added, the flask agitated and a sample withdrawn immediately, then at intervals over 4 h. The samples were frozen for later analysis of nitrite and nitrate concentration. Nitrate uptake rate was calculated from the decline in nitrate concentration in the seawater medium over time using linear regression.

### Tissue nutrient and NR analysis

NRA was estimated using an assay on seaweed extracts described by Young et al. (2005). Internal inorganic N pools were extracted by placing approximately 50 mg samples of ground frozen thallus in boiling tubes with 20 ml room temperature milli-Q water (Millipore, Watford, Herts, UK), heating in a boiling water bath for 45 min, cooling to room temperature and filtering through Whatman GF/A filters (Young et al. 2007a). Nitrate was analysed by Cd column reduction followed by spectrophotometric measurement of nitrite, and ammonium was estimated by the phenol-hypochlorite method, both according to

Parsons et al. (1984). Total N and C content of thalli was measured in ground, oven-dried samples using a Carlo Erba 1500 NC elemental analyzer (CE Elantech, Lakewood, NJ) using acetanilide as a standard (Young et al. 2007a).

### Statistical analysis

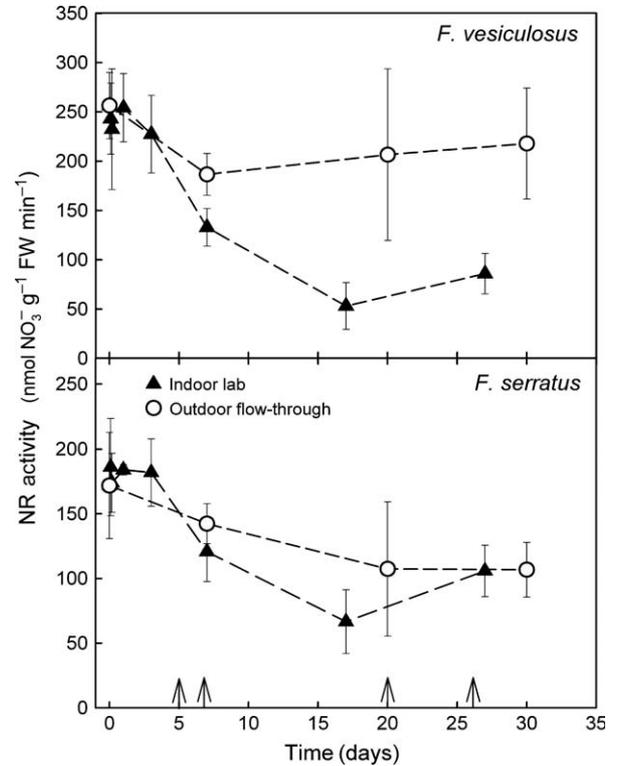
Preliminary samples were used to test the variance in NRA between and within thalli. An *F*-test showed that the variability of NRA in six tips from each thallus was not significantly different from that of NRA in tips from six different individuals sampled over 2 days ( $P > 0.05$ ). Thereafter, all tips were treated as independent for statistical comparisons (Young et al. 2007b). Changes in NRA and internal nutrient concentrations in response to experimental manipulations were compared using one-way ANOVA for N treatment or two-way ANOVA with N treatment and time as variables (SIGMASTAT v. 3.1; Systat Software Inc, Chicago, IL).

### Results

#### Tank storage, N deprivation and N resupply in *Fucus*

*Fucus serratus* and *F. vesiculosus* maintained in indoor tanks showed a decline in NR between 5 and 17 days (Fig. 1;  $P < 0.03$ ) but some recovery after resupply with nitrate on day 20. In contrast, *F. serratus* and *F. vesiculosus* maintained in outdoor tanks exposed to ambient light and temperature conditions and supplied with flow-through natural seawater showed no statistically significant decline in NR over 1 month (Fig. 1;  $P > 0.05$  for both species).

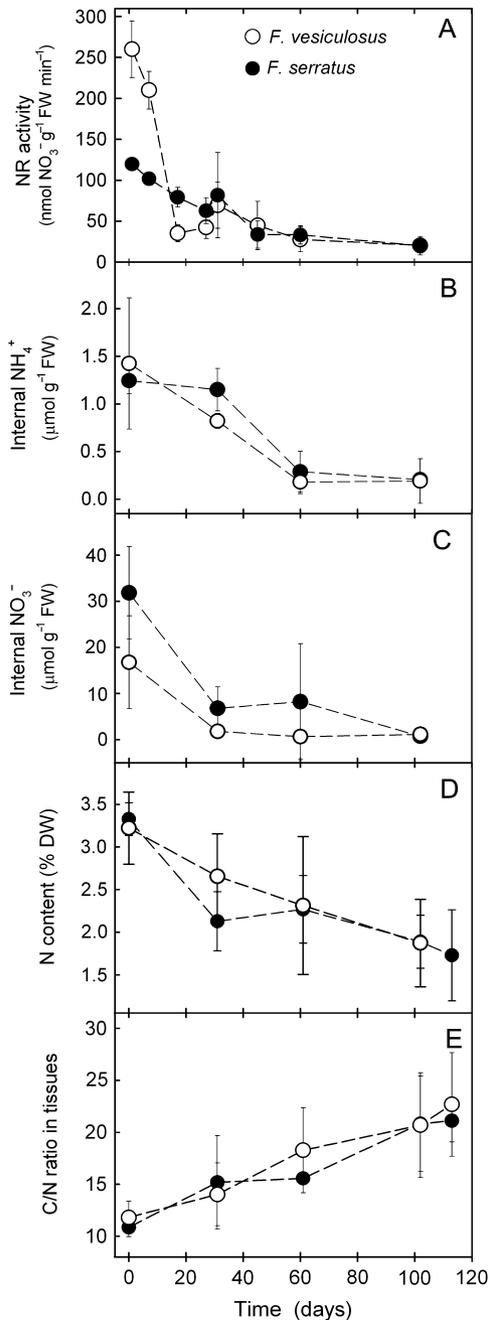
In *Fucus* thalli, there were substantial changes in NR and N content during 15 weeks of N deprivation in laboratory culture. NRA in *F. vesiculosus* declined sharply within the first 2 weeks of N deprivation ( $P < 0.001$ ), whereas in *F. serratus*, there was a gradual decline over the full 15 weeks of the experiment ( $P < 0.004$ ; Fig. 2A). In both species, even after being deprived of external nitrate for 15 weeks, a low level of NRA was maintained within the thalli [ $\geq 20$  nmol nitrate  $\text{g}^{-1}$  fresh weight (FW)  $\text{min}^{-1}$  for both species]. Internal nitrate and ammonium concentrations and total N content [as % dry weight (DW)] decreased dramatically (Fig. 2B–D). C content (as % C) did not change significantly ( $P > 0.05$ ), so the C : N ratio in the thalli increased during the 15 weeks of N deprivation ( $P < 0.025$ ) (Fig. 2E). When calculating for tissue subsamples removed throughout 15-week experiment, estimates of net thallus biomass increase were approximately 55% in *F. serratus* and approximately 80% in *F. vesiculosus*. Despite declines in N content,  $F_v/F_m$  in *F. serratus* and *F. vesiculosus* did not change significantly over the



**Fig. 1.** Changes in NRA in *Fucus vesiculosus* and *Fucus serratus* during incubation in outdoor continuous flow-through tanks at Portaferry (circles) or indoor laboratory tanks with aeration (triangles). Points are means  $\pm$  SD,  $n = 4$ . In indoor cultures, seawater medium was changed, and nitrate and phosphate were added to tanks on days 0, 5, 7, 20 and 27 (arrows).

15 weeks of N deprivation ( $P > 0.05$ );  $0.689 \pm 0.049$  to  $0.679 \pm 0.018$  in *F. serratus* and  $0.687 \pm 0.026$  to  $0.688 \pm 0.068$  in *F. vesiculosus*.

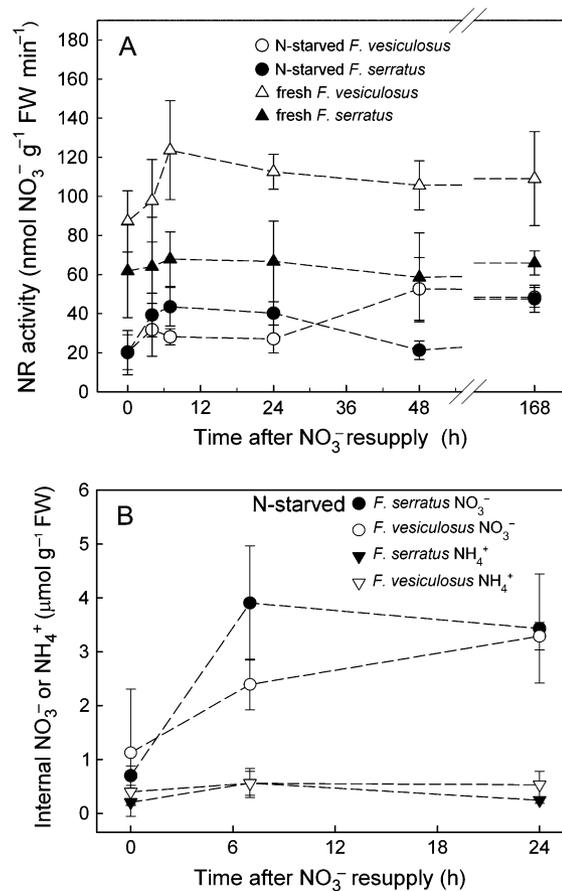
Following resupply of nitrate to the seawater medium after 15 weeks of N deprivation, NRA increased in *F. vesiculosus* and *F. serratus* thalli (Fig. 3;  $P < 0.003$  over 7 days). However, 1 week after nitrate resupply, NRA had not recovered to levels prior to N deprivation (cf. Fig. 2A). In *F. serratus*, nitrate resupply resulted in increases in NRA ( $P < 0.03$ ) and in internal nitrate concentration ( $P < 0.03$ ) over just 7 h (Fig. 3B), but increases in *F. vesiculosus* occurred more slowly. NRA in *F. serratus* had declined by 48 h but increased again by day 7. In both species, there were no significant changes in internal ammonium concentrations ( $P > 0.05$ ; Fig. 3B) or in total thallus N content and C : N ratio 1 week after nitrate resupply (data not shown). In contrast to N-starved thalli, changes in NRA over 7 days in freshly collected thalli also supplied with a  $100 \mu\text{M}$  nitrate pulse were not statistically significant ( $P > 0.05$ ) (Fig. 3A).



**Fig. 2.** Changes in N metabolism in *Fucus vesiculosus* (filled symbols) and *Fucus serratus* (open symbols) during 15 weeks of N deprivation. (A) NRA, (B) internal ammonium concentration, (C) internal nitrate concentration, (D) N content (% DW) and (E) C : N (mol mol<sup>-1</sup>) content in thalli. Symbols are means  $\pm$  SD, n = 3.

### Effects of inorganic N sources

When thalli were exposed to twice-daily additions of different sources inorganic N, nitrate and ammonium concentrations in the elevated N treatments fluctuated as



**Fig. 3.** Changes in *Fucus vesiculosus* and *Fucus serratus* following resupply of 100  $\mu$ M nitrate to thalli previously deprived of N for 15 weeks (Fig. 2). (A) Changes in NRA compared with freshly collected thalli (triangles) also supplied with 100  $\mu$ M nitrate. (B) Changes in internal nitrate (circles) or ammonium (triangles) pools following nitrate resupply. All points are means  $\pm$  SD, n = 4.

uptake by the thalli removed nutrients from the seawater medium between additions. In the elevated ammonium treatment, ammonium concentration ranged from 0 to 75  $\mu$ M, but nitrate concentration was constantly  $<0.5$   $\mu$ M; in the elevated ammonium plus nitrate treatment, ammonium fluctuated between 0 and 100  $\mu$ M and nitrate between 0 and 150  $\mu$ M; in the elevated nitrate treatment, ammonium was constantly  $<0.2$   $\mu$ M, whereas nitrate was 5–100  $\mu$ M; however, in the control treatment without additions, ammonium was  $<0.1$   $\mu$ M and nitrate was  $<2$   $\mu$ M. Seawater nitrite concentrations were  $<0.6$   $\mu$ M in all treatments throughout the experiment.

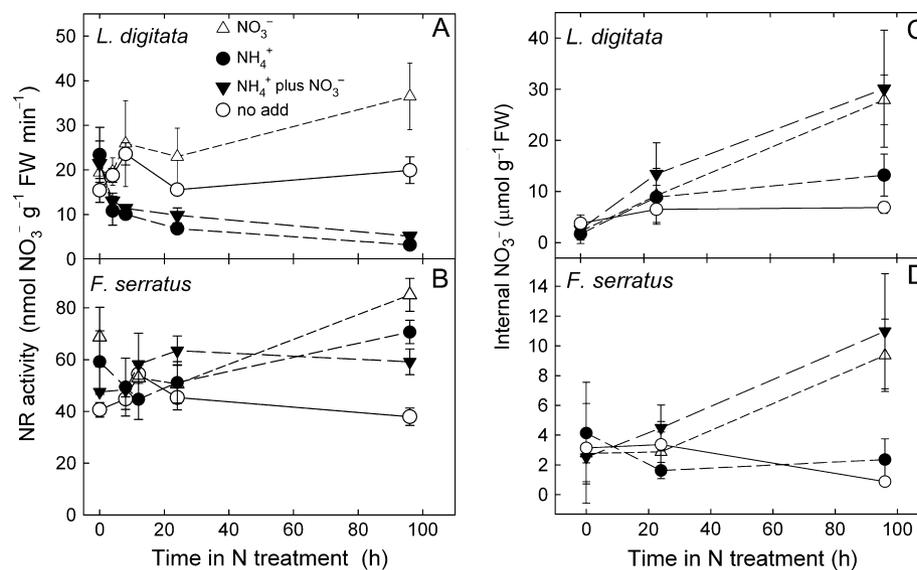
NRA in *L. digitata* was decreased in elevated ammonium and ammonium plus nitrate treatments (Fig. 4A), with significant declines within the first 4–24 h after transfer to elevated N treatments ( $P < 0.04$ ). When

*L. digitata* was exposed to only nitrate or no N additions, there was no change in NRA ( $P > 0.05$ ) (Fig. 4A), but after 96 h, the NRA was significantly lower in both elevated ammonium and ammonium plus nitrate treatments ( $P < 0.03$ ). In contrast to *L. digitata*, NRA in *F. serratus* showed no significant decline in NRA in response to elevated ammonium, but after 96 h, the elevated nitrate treatment had higher NRA ( $P < 0.037$ ) than the other treatments (Fig. 4B).

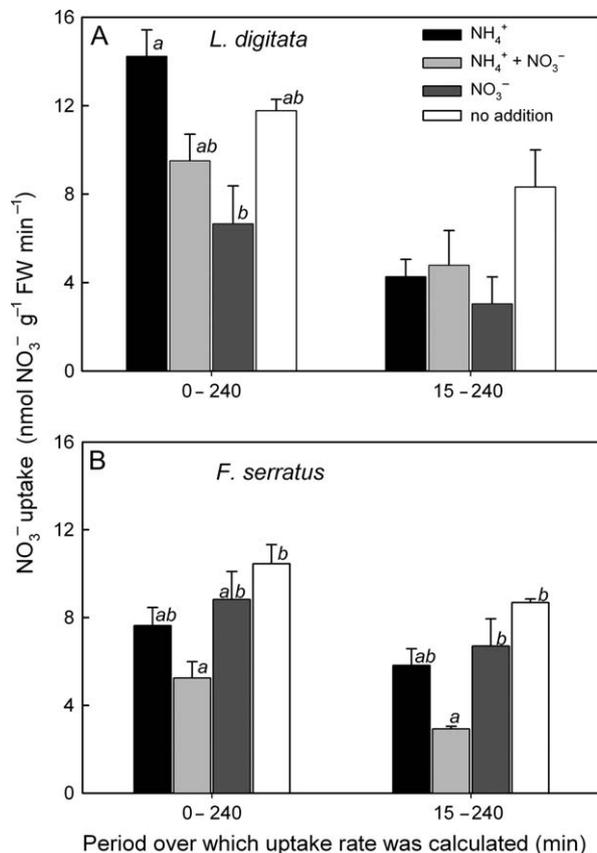
Elevated N treatment incubations resulted in increases in internal thallus nitrate pools, which reached 2.5 times higher concentrations in *L. digitata* than in *F. serratus* (Fig. 4C, D). After 96 h, *L. digitata* thalli from the elevated nitrate treatments had higher internal nitrate concentrations than at the beginning of the experiment ( $P < 0.009$ ; Fig. 4C). Elevated ammonium did not result in internal nitrate concentrations higher than controls in either species ( $P > 0.05$ ). In all thalli, internal nitrate concentrations were approximately 10 times higher than internal ammonium, which did not change with N treatment ( $P > 0.05$ ). Internal nitrite was  $< 0.1 \mu\text{mol g}^{-1}$  FW in all treatments. By the end of the experiment, total thallus N content in *F. serratus* ( $1.54 \pm 0.206\%$  DW) was not significantly different between treatments ( $P > 0.05$ ), but in *L. digitata*, treatment influenced N content – elevated ammonium plus nitrate (2.37% DW) was higher than elevated nitrate (1.81% DW) and no additions (1.42% DW) ( $P < 0.02$ ); elevated ammonium (2.32% DW) was higher than elevated nitrate and no

additions ( $P < 0.03$ ). C content of all thalli did not change significantly with time or treatment ( $P > 0.05$ ). Total thallus C : N content ratios did not change in *F. serratus* but decreased significantly from  $>18$  to  $<12$  in *L. digitata* in the elevated N treatments only ( $P < 0.001$ ).

*Laminaria digitata* showed slightly higher nitrate uptakes than *F. serratus* (Fig. 5), particularly over the first 15 min; in *L. digitata*, there was a more pronounced higher uptake rate in the 0- to 240-min than in the 15- to 240-min time period. Uptake rates over 15 min (based on two time points) were  $97\text{--}300 \text{ nmol g}^{-1} \text{ FW min}^{-1}$  for *L. digitata* and  $62\text{--}140 \text{ nmol g}^{-1} \text{ FW min}^{-1}$  for *F. serratus*. Elevated ammonium treatments did not result in reduced nitrate uptake capacity in either species. *L. digitata* thalli exposed to elevated ammonium had higher nitrate uptake rates over 240 min than thalli exposed to elevated nitrate ( $P < 0.006$ ). However, when the initial 15 min of uptake was excluded, there were no significant differences among the uptake rates in the elevated N treatments ( $P > 0.05$ ; Fig. 5A). *F. serratus* thalli from elevated ammonium plus nitrate showed significantly lower nitrate uptake rates than control thalli ( $P < 0.03$ ). When mass-specific nitrate uptake rates were recalculated on the basis of thallus N content, N-specific nitrate uptake rates were  $0.2\text{--}1.0 \text{ nmol NO}_3^- \text{ mg}^{-1} \text{ N min}^{-1}$  (equivalent to  $0.168\text{--}0.84 \text{ h}^{-1}$ ) for both species. This resulted in N-specific uptake rates over both the 0- to 240-min and the 15- to 240-min time periods for the no additional N treatment to be higher than at least one elevated N treatment in both species.



**Fig. 4.** Changes in *Laminaria digitata* and *Fucus serratus* over 4 days incubation with four different inorganic N treatments – (1) elevated nitrate (open triangles); (2) elevated ammonium (filled circles); (3) elevated ammonium plus nitrate (filled triangles); and (4) no N addition controls (open circles). (A) and (B) NRA. (C) and (D) Internal thallus nitrate concentration, note different nitrate scales for the two species. Points are mean  $\pm$  SD,  $n = 4$ .



**Fig. 5.** Nitrate uptake rates measured in (A) *Laminaria digitata* and (B) *Fucus serratus* thalli after 4 days incubation in four different inorganic N treatments (Fig. 4). Bars represent mean nitrate uptake rates measured over 0–240 or 15–240 min  $\pm$  SE,  $n = 3$ . Bars marked with different lowercase letters indicate that means are significantly different ( $P < 0.05$ ).

## Discussion

### Tank cultivation

Maintenance of intertidal brown algae in flow-through tanks exposed to ambient irradiance, temperature and moving natural Lough water is a good option for longer term experiments focused on seaweed N metabolism, as NRA did not significantly decline over 1 month. However, thalli maintained in indoor tanks showed some changes in NRA over 1 month, suggesting that this is a good option only for shorter term experiments. Differences between indoor and outdoor culture include light spectrum and water movement and turnover. Decline in NR in thalli maintained in the indoor tanks between day 7 and 20 may also be related to lack of nitrate, as NRA increased after nitrate addition on day 20, whereas thalli in outdoor tanks were exposed to a more constant nitrate concentration in the Lough water. In nature, these species

experience tidal cycles that were also not replicated in either tank set-up. Despite the fact that *Fucus* grows higher in the intertidal and so is adapted to longer emersion periods during each tidal cycle than the lower subtidal–intertidal *L. digitata*, *Fucus* species were more resilient to long-term submersion in tank culture experiments. Thalli of *F. serratus* and *F. vesiculosus* were intact and physiologically active after 15 weeks in indoor tank culture, whereas *L. digitata* thalli disintegrated after less than a month of immersion. *F. vesiculosus* grows permanently submerged in non-tidal regions of the northern Baltic Sea (Nygård and Dring 2008) where *Laminaria* species are lacking (Nielsen et al. 1995). This suggests that *Fucus* may have higher tolerance to continuous submergence, as well as the lower salinity in those regions, than *Laminaria* spp. However, previous studies have shown that with the right light regime, *Laminaria* spp. can do well in long-term flow-through outdoor culture, so this disintegration in laboratory cultures may be associated with conditions stimulating reproductive activity resulting in fragmentation (Pang and Lüning 2004). Bacteria associated with alginate breakdown (Norderhaug et al. 2003) could also hasten *Laminaria* spp. degradation in low-flow conditions.

### Effects of long-term N deprivation on N metabolism and photosynthesis

Long-term N deprivation had clear effects on N metabolism with declines in total N and internal nitrate and ammonium pools and NRA. The most dramatic declines in NRA were coincident with depletion of internal nitrate over the first 6 weeks of N deprivation. In unicellular algae, NRA requires the presence of nitrate to maintain synthesis of the NR enzyme (Vergara et al. 1998; reviewed by Berges 1997). However, in *F. serratus* and *F. vesiculosus*, some NRA was maintained after several weeks of nitrate deprivation and minimal internal nitrate concentrations when one might expect NRA is not required. This may be adaptive in an intertidal environment to allow rapid assimilation of intermittently available nitrate. NRA in brown algae may depend on both an environmentally inducible as well as a constitutively produced form of the enzyme, as occurs in higher plants (Lillo 2008, Yang and Midmore 2005). In response to N starvation, internal total N and nitrate pools were depleted more slowly in *Fucus* than in the N-starved rhodophyte *Gracilaria tikvahiae* (Hwang et al. 1987). N turnover is also faster in *Ulva* (Teichberg et al. 2007). This difference may reflect greater N storage capacity in the more robust thalli of the furoids. The critical N content below which growth of *F. vesiculosus* is compromised has been estimated at approximately 1.7% N DW, which is lower than that for some other

macroalgal species (Pedersen and Borum 1996). The thalli only reached these low levels at the end of 15 weeks of N deprivation. However, during a natural seasonal cycle, thalli do reach similarly low, potentially growth-reducing N content in the late summer and autumn (Young et al. 2007a). Thalli used for the experiments on the effects of elevated ammonium and nitrate (below) were collected in July and had much lower initial N content than in March, at the beginning of the N-deprivation experiment. Estimated growth does not account for all the loss of tissue N content during 15 weeks of N deprivation – N content halved, but thallus mass increased by only 55–80%. Therefore, there may have been some leakage of organic N from the thallus, particularly in the more advanced stages of N deprivation (Tyler et al. 2001).

Although low N content likely impaired growth, the  $F_v/F_m$  in *F. serratus* and *F. vesiculosus* were maintained despite other changes over 15 weeks of N deprivation. This contrasts with unicellular algal cells in which  $F_v/F_m$  declines over <24 h when deprived of N (Geider et al. 1993, Young and Beardall 2003). Large brown macroalgae can store considerable amounts of inorganic N internally (Gómez and Wiencke 1998, Hurd et al. 1996, Poeschel and Korb 2001, Wheeler and Srivastava 1984); up to 80  $\mu\text{mol}$  nitrate  $\text{g}^{-1}$  FW in *F. vesiculosus* (Young et al. 2007a). Photosynthetic quantum yield in these species is known to respond rapidly to other stressors (e.g. high irradiance: Dring et al. 2001; Figueroa et al. 2003), so acclimation over several weeks of N deprivation clearly involved redistribution of stored N to maintain the photosynthetic apparatus and quantum yield. Translocation of N has been demonstrated in *Laminaria* (Davison and Stewart 1983) and of organic C in *Fucus* (Diouris 1989), and there is indirect evidence for N translocation in *F. vesiculosus* (Honkanen and Jormalainen 2002). Therefore, acclimation to long-term N deprivation in *Fucus* species could involve some redistribution of N within the thallus via translocation. N deprivation has also been shown to have little effect on  $F_v/F_m$  in a green macroalga (Malta et al. 2005) and some polar brown macroalgae but resulted in rapid decline of  $F_v/F_m$  in temperate *Laminaria saccharina* after 10 weeks of N deprivation (Korb and Gerard 2000). Marine brown algae in high latitudes, including *Laminaria* and *Fucus* species, have to survive low winter temperatures and irradiance accompanied by high nitrate availability but, in summer, may be exposed to high irradiance and low nitrate concentrations (Henley and Dunton 1997, Korb and Gerard 2000, Young et al. 2007a). The ability to maintain photosynthetic function despite seasonal energy and macronutrient deprivation is an important adaptation of long-lived brown macroalgae from highly seasonal temperate habitats.

### Recovery from N deprivation following $\text{NO}_3^-$ resupply

Despite 15 weeks of N deprivation of *F. serratus* and *F. vesiculosus*, the thalli responded rapidly to resupply of nitrate, suggesting that the thalli are well adapted to opportunistic uptake of newly available nitrate, even after prolonged seasonal N deprivation (Chapman and Craigie 1977, Young et al. 2007a). Inorganic N pools (mostly nitrate) represented up to 44% of total N pools in N-replete thalli (calculated from Fig. 2B–D). Unassimilated nitrate as well as proteins and free amino acids are important N storage forms in macroalgae (Korb and Gerard 2000, McGlathery et al. 1996, Naldi and Wheeler 1999, Poeschel and Korb 2001, Young et al. 2007a). Increases in NRA during 4–7 h after resupply of nitrate are consistent with de novo synthesis of NR protein (Vergara et al. 1998). The lack of recovery to prestarvation NRA within the week following nitrate resupply (Fig. 2A), and no measurable change in total thallus N content, may be because the single 100  $\mu\text{M}$  nitrate pulse was rapidly taken up and ‘diluted’ into thallus tissue. Assuming that the entire 100  $\mu\text{M}$  nitrate added was taken up by approximately 100 g thallus tissue in each 20-l tank, and that NRA was the same throughout the thallus mass, the NRA measured in *F. serratus* tips at 4 h after nitrate resupply would have been sufficient to reduce all the added nitrate in 8.3 h, thus reducing the stimulus for further NR protein synthesis. Although the in vitro NR assay used could potentially overestimate in vivo activity (see below), this represents an apparent excess nitrate assimilation capacity. Despite this capacity, the thalli stored some unassimilated nitrate, with significant increases in internal nitrate, from approximately 1 to >3  $\mu\text{mol}$   $\text{g}^{-1}$  FW 24 h after nitrate resupply. Assuming even distribution within the thalli, this represents approximately 15% of the 100  $\mu\text{M}$  added, so approximately 85% was assimilated into ammonium and organic forms.

Responses to the different inorganic N treatments suggest that N deprivation could have stimulated nitrate uptake rates, at least over a number of days (Fig. 5). Although it is unlikely that elevated nitrate uptake capacity will be maintained under longer term N deprivation (Parslow et al. 1984), the maximum nitrate uptake rate measured in healthy *F. serratus* (approximately 10  $\text{nmol}$  nitrate  $\text{g}^{-1}$  FW  $\text{min}^{-1}$ ; Fig. 5B) was half the minimum nitrate assimilation capacity (NRA) in *F. serratus*, which had been N deprived for 15 weeks (20  $\text{nmol}$  nitrate  $\text{g}^{-1}$  FW  $\text{min}^{-1}$ ; Fig. 2A). Intertidal brown algae apparently maintain an excess nitrate reduction capacity (NRA), even when deprived of a source of nitrate to assimilate. A similar conclusion was made based on seasonal comparison of nitrate

assimilation and photosynthesis rates in intertidal brown algae (Young et al. 2007a).

Nitrate reduction, catalysed by NR, is often considered the rate-limiting step in algal nitrate assimilation (Berges 1997, Davison and Stewart 1984, Lartigue and Sherman 2005). In these brown macroalgae, N assimilation may well be limited by other factors, such as energy supply (Henley and Dunton 1997, Young et al. 2007a, 2007b). It may be that, after 15 weeks of N deprivation, photosynthetic enzyme pools (e.g. Rubisco) were low (Wheeler and Weidner 1983) so that the supply of C skeletons via photosynthesis required for N assimilation may be limited (Flynn 1991). There was no evidence for uptake and reduction of nitrate with subsequent excretion of nitrite. Furthermore, as the internal concentrations of nitrite and ammonium remained low and unchanged following nitrate resupply, it is likely that further assimilation of nitrite to ammonium and into amino acids, catalysed by nitrite reductase and glutamine synthetase, was not limiting overall nitrate assimilation. NRA is apparently present in excess, but nitrate reduction still represents a key control step N assimilation in these brown macroalgae. Our *in vitro* assay may overestimate *in vivo* activity (Young et al. 2005) because of post-translational regulation (e.g. by phosphorylation) of enzyme activity, which may be lost during extraction (Huber et al. 1992, Lillo 2008). *In vivo* reduction of nitrate may also be limited by compartmentalization of nitrate into cellular compartments isolated from NR (e.g. the vacuole, Granstedt and Huffaker 1982) until assimilation into amino acids is required or can be sustained by supply of energy and/or C skeletons (Flynn 1991).

### Effects of exposure to ammonium and nitrate on nitrate uptake and assimilation

The availability of inorganic N as nitrate or ammonium influenced NRA in *L. digitata* and *F. serratus*. In both species, elevated nitrate alone stimulated NRA, but even when nitrate concentration was low (<2  $\mu\text{M}$ ), some NRA was maintained. However, NRA in *L. digitata* and *F. serratus* responded differently to elevated ammonium. Ammonium suppression of NRA and decline in NR protein has been observed in several algal species and higher plants (Balandin and Aparicio 1992, Berges et al. 1995, Nicodemus et al. 2008, Vergara et al. 1998, Weidner and Kiefer 1981). In some plant species, ammonium can inhibit or promote NRA, depending on the tissues (Leleu and Vuylsteker 2004). The current study shows the rapid decline of *L. digitata* NRA in response to ammonium on a time scale (4–8 h) compatible with a decline in NR protein synthesis or increase in protein

degradation. The mechanism of ammonium inhibition of NR is unknown, but it has been suggested that it occurs via a metabolite of ammonium assimilation, e.g. glutamine (Flynn 1991, Vergara et al. 1998), or indirectly by inhibiting nitrate uptake (Collos 1989). Despite ammonium inhibition of NRA, nitrate uptake capacity was not suppressed by ammonium, so effects on NRA in *L. digitata* did not occur via suppression of nitrate uptake. Furthermore, the presence of nitrate in addition to the ammonium did not prevent ammonium suppression of NRA in *L. digitata*, suggesting that ammonium per se has a strong effect either via direct inhibition of NR enzyme activity or via protein synthesis and/or degradation. None of these inhibition steps appeared to be operating in *F. serratus*, and a low level of NRA maintained in *L. digitata* thalli exposed to elevated ammonium was also insensitive to ammonium inhibition.

The mass-specific nitrate uptake rates by *L. digitata* and *F. serratus* were lower than uptake rates in *Ulva* (Naldi and Viaroli 2002), but when expressed in relation to thallus N content, N-specific nitrate uptake rates (0.168–0.84  $\text{h}^{-1}$ ) were high compared with those in microalgae (0.03–0.06  $\text{h}^{-1}$ ; Cochlan and Harrison 1991, Parslow et al. 1984). Nitrate uptake has been shown to be inhibited by ammonium in a range of algae (Collos 1989, Dortch 1990, Lobban and Harrison 1997, Rees et al. 2007); however, ammonium suppression of nitrate uptake rate was not observed in *F. serratus* or *L. digitata*. Absence of ammonium suppression has also been reported in the brown algae *Laminaria groenlandica* (Harrison et al. 1986) and *Macrocystis integrifolia* (Wheeler and Srivastava 1984). Although ammonium did not suppress nitrate uptake, there was an effect of 'N history' in *L. digitata* and *F. serratus*, as in other algae (Fong et al. 1994, Naldi and Viaroli 2002, Pedersen and Borum 1996, Young and Beardall 2003). In *F. serratus*, the lower nitrate uptake rate in thalli exposed to elevated ammonium plus nitrate suggests a downregulation of uptake capacity, associated with nitrate transporters (Hildebrand and Dahlin 2000), when the thalli are well supplied with inorganic N (Lartigue and Sherman 2005, Naldi and Viaroli 2002). Higher nitrate uptake rates were calculated over the first 15 min of exposure to nitrate than over 15–240 min. This 'surge' uptake was more pronounced in *L. digitata* than in *F. serratus* and is an adaptation for competitive uptake of inorganic N in a habitat with variable N supply (Harrison et al. 1986, Pedersen 1994, Thomas and Harrison 1987). Throughout the seasonal cycle, low concentrations of ammonium were always present in Strangford Lough water (1–3  $\mu\text{M}$ ; Young et al. 2007a), so maintenance of high nitrate uptake rates, and some NRA, in the presence of ammonium is probably an adaptation to maintaining

nitrate uptake and assimilation capacity under normal field conditions for these intertidal brown algae.

There is evidence for epiphytic nitrifying bacteria associated with macroalgae in culture (Hernández et al. 2006), the activity of which may influence pools of different inorganic N forms available on the surface of the thallus. However, the nitrate concentration measured in elevated ammonium treatment tanks was consistently  $<0.5 \mu\text{M}$ , so any microbial nitrate production was either extremely low or nitrate released by nitrification was taken up immediately.

### Differences between *Fucus* and *Laminaria*

The differences between *L. digitata* and *Fucus* species in response to inorganic N availability may be related to differences in the evolution of N acquisition strategies and/or to the relative positions of the species in the intertidal environment. Previous studies have associated higher position in the intertidal with higher internal nitrate pools (Phillips and Hurd 2003), higher NR activities (Murthy et al. 1986, Young et al. 2007b) and elevated nutrient uptake capacity, which may compensate for the shorter periods of emersion (Hurd and Dring 1990, Phillips and Hurd 2004, Thomas et al. 1987). However, this did not explain differences in nitrate uptake by the subtidal to lower intertidal *L. digitata* and mid-intertidal *F. serratus*. Greater 'surge' uptake observed in *L. digitata* might be expected in a species found higher in the intertidal where it is isolated from nutrient source for longer each day and would benefit from a more rapid uptake of nutrients during shorter periods of immersion. Although the biomass of *L. digitata* in each 20-l seawater tank was only 70% of that of *F. serratus*, nearly three-fold higher internal nitrate concentrations were observed in *L. digitata* than in *F. serratus*. These differences between *L. digitata* and *F. serratus* may relate to distinct N acquisition strategies, as also observed in distinct light regulation of NRA in *Fucus* and *Laminaria* species (Young et al. 2007b). *Laminaria* species have been described as having a 'storage specialist' strategy, which enables uptake and storage of inorganic N during high winter availability to support summer growth under higher irradiance but limiting nitrate supply (Henley and Dunton 1997, Korb and Gerard 2000, Young et al. 2007a). The ability to take up nitrate rapidly and to store high concentrations, even in the presence of or after recent exposure to ammonium, and to maintain apparently constitutive and excess NRA, are physiological adaptations that allow these intertidal macroalgae to sequester inorganic N efficiently and to support growth in a seasonally variable, intertidal habitat.

**Acknowledgements** – E. B. Y. was supported by a postdoctoral NERC UK grant (GR3/12454) to J. A. B and M. J. D. This study was facilitated by a summer grant from the British Phycological Society to E. B. Y. and dedicated laboratory assistance from Cristina Molina. C and N analysis was carried out by Brian Stewart from Agri-Food and Biosciences Institute Northern Ireland, with sample preparation assistance from Dan Franklin. Two anonymous reviewers provided valuable suggestions.

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