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Nitrate reductase activity in macroalgae and its vertical distribution in macroalgal epiphytes of seagrasses

E. B. Young[1,3,*], P. S. Lavery[2], B. van Elven[2], M. J. Dring[1], J. A. Berges[1,3]

[1]Marine Systems Group, School of Biology and Biochemistry, Queen's University of Belfast, Belfast BT9 7BL, UK
[2]Centre for Ecosystem Management and School of Natural Sciences, Edith Cowan University, Joondalup, Western Australia 6027, Australia

[3]*Present address:* Department of Biological Sciences, University of Wisconsin-Milwaukee, PO Box 413, Milwaukee, Wisconsin 53201, USA

ABSTRACT: Macroalgal epiphytes within seagrass meadows make a significant contribution to total primary production by assimilating water column N and transferring organic N to sediments. Assimilation of NO_3^- requires nitrate reductase (NR, EC 1.6.6.1); NR activity represents the capacity for NO_3^- assimilation. An optimised *in vitro* assay for determining NR activity in algal extracts was applied to a wide range of macroalgae and detected NR activity in all 22 species tested with activity 2 to 290 nmol NO_3^- min^{-1} g^{-1} frozen thallus. With liquid-N_2 freezing immediately after sample collection, this method was practical for estimating NR activity in field samples. Vertical distribution of NR activity in macroalgal epiphytes was compared in contrasting *Posidonia sinuosa* and *Amphibolis antarctica* seagrass meadows. Epiphytes on *P. sinuosa* had higher mass-specific NR activity than those on *A. antarctica*. In *P. sinuosa* canopies, NR activity increased with distance from the sediment surface and was negatively correlated with [NH_4^+] in the water but uncorrelated with [NO_3^-]. This supported the hypothesis that NH_4^+ released from the sediment suppresses NR in epiphytic algae. In contrast, the vertical variation in NR activity in macroalgae on *A. antarctica* was not statistically significant although there was a weak correlation with [NO_3^-], which increased with distance from the sediment. Estimated capacities for NO_3^- assimilation in macroalgae epiphytic on seagrasses during summer (24 and 46 mmol N m^{-2} d^{-1} for *P. sinuosa* and *A. antarctica*, respectively) were more than twice the estimated N assimilation rates in similar seagrasses. When the estimates were based on annual average epiphyte loads for seagrass meadows in other locations, they were comparable to those of seagrasses. We conclude that epiphytic algae represent a potentially important sink for water-column nitrate within seagrass meadows.

KEY WORDS: Nitrate reductase · Macroalgae · Seagrass epiphytes · Dissolved inorganic nitrogen · Ammonium · Nitrate · Irradiance

INTRODUCTION

Macroalgal epiphytes within seagrass meadows make a significant contribution to biomass (Paling & McComb 2000) and total primary production of a meadow (Cambridge & Hocking 1997), with production even exceeding that of their seagrass hosts, particularly under low light (Libes 1986). The detrimental effect of excessive epiphytic macroalgal growth on seagrass viability is well documented, particularly in areas with increasing anthropogenic nutrient inputs to coastal waters (Silberstein et al. 1986, Hauxwell et al. 2001, McGlathery 2001). However, in oligotrophic waters epiphytic macroalgae can play an important role in ecosystem function by contributing to the capture of scarce N resources from the water column and

*Email: ebyoung@uwm.edu

the transfer of relatively N-rich organic matter to the sediments of seagrass meadows, promoting nutrient cycling (reviewed by McGlathery 2001).

Coastal waters off Western Australia have very low concentrations of inorganic N, mostly in the form of nitrate, which in summer is typically <0.6 µM (Johannes et al. 1994), yet they support abundant seagrass communities with diverse macroalgal epiphyte assemblages (Lavery & Vanderklift 2002). The contribution of epiphytes to the total N assimilation by seagrass meadows, and the extent to which epiphytic macroalgae compete for water column inorganic nitrogen, are poorly understood. The nitrogen requirements of seagrass meadows are also met by remineralisation of organic nitrogen in sediments and subsequent release of ammonium, much of which is rapidly assimilated by the seagrass and epiphytic communities and thus not released to the overlying water column (Lee & Dunton 1999).

Hauxwell et al. (2001) measured a gradient in ammonium concentration in a *Zostera marina* canopy, with highest concentrations closest to the sediment surface. Ammonium is taken up rapidly, and often preferentially over nitrate, by both seagrasses and macroalgae (Thomas & Harrison 1985, Paling & McComb 1994, Pedersen et al. 1997). Although inorganic N regenerated in the sediments is known to be important in supporting seagrass production (Lee & Dunton 1999), little is known about the relative importance of nitrate and ammonium for epiphytic algae, or the role of ammonium released from the sediment in determining nitrate use by algal epiphytes within the meadow. Dissolved inorganic N concentrations can be misleading because of rapid N uptake and turnover, so additional measures of nitrate assimilation are needed.

The capacity of algae to assimilate nitrate depends upon the enzyme nitrate reductase (NR, EC 1.6.6.1), which reduces nitrate to nitrite. NR activity is regulated in response to nitrate availability, is inhibited by ambient ammonium and is regarded as the rate-limiting step in uptake and assimilation of nitrate into amino acids (Dortch 1990, Solomonson & Barber 1990). Measurement of NR activity has been reported in relatively few macroalgal species (see Hurd et al. 1995), but studies have observed a strong correlation between NR activity and rates of nitrate incorporation both in phytoplankton (Berges et al. 1995) and in macroalgae (Davison et al. 1984). We aimed to use NR activity to estimate the nitrate assimilating capacity of epiphytic algae in seagrass meadows. While NR allows epiphytes to use nitrate from the water column, the gradient in ammonium released from seagrass sediments may suppress NR expression in macroalgae closer to the sediment and thus influence the form of inorganic N being taken up and assimilated by macroalgae depending on vertical distance from the sediment.

Measurement of NR activity can be difficult and some earlier papers reported low or no activity using an *in vitro* assay for algal extracts (e.g. Thomas & Harrison 1988, Corzo & Niell 1991) and seagrass tissues (Touchette & Burkholder 2001). Our preliminary results also failed to detect NR activity in seagrass leaves with this method. An alternative *in situ* assay measuring NR activity can yield higher activities (Corzo & Niell 1991, Lartigue & Sherman 2002), but *in situ* assays require tissue permeabilisation, which can confound interpretation (see Thomas & Harrison 1988, Corzo & Niell 1991, Hurd et al. 1995). Hurd et al. (1995) reported an improved *in vitro* method to assay NR activity which they tested on extracts of 6 macroalgal species. This method provides an algal-specific method for determining NR activity in seagrass epiphytes. Because the assay measures the activity of NR with substrates (i.e. NO_3^- and NADH [nicotinamide dinucleotide diphosphate, reduced form]) at saturating concentrations (i.e. a conventional V_{max} assay), the activity measured is normally considered to represent a potential maximum. However, as a rate-limiting enzyme, NR may often be substrate-saturated, so NR activity may correlate quite closely with *in vivo* incorporation rates (e.g. Berges et al. 1995).

In this study, we aimed to establish a robust *in vitro* NR assay and demonstrate broad applicability in a wide range of algal taxa from the northern and southern hemispheres. Using the Hurd et al. (1995) NR assay, optimised for our species, we tested the hypothesis that ammonium released from seagrass sediments suppresses NR activity in macroalgal epiphytes within seagrass canopies. With this aim, we determined the vertical distribution of NR activity in macroalgal epiphytes in relation to vertical profiles of ammonium and nitrate concentration within 2 contrasting seagrass meadow types. We also evaluated the potential contribution of epiphytic algae to total inorganic N assimilation by seagrass meadows by using NR as an estimate of nitrate assimilation capacity.

MATERIALS AND METHODS

Sampling of macroalgal epiphytes. Stems of *Amphibolis antarctica* ([Labill.] Sonder & Aschers. *ex* Aschers) and leaves of *Posidonia sinuosa* (Cambridge & Kuo) with resident macroalgal epiphyte communities were collected from monospecific seagrass meadows in Shoalwater Bay, south of Perth, Western Australia (31° 17′ S, 115° 42′ E) in February 2001 at a depth of 2 to 5 m using SCUBA. Leaves or stems were divided into top, middle and lowest portions (corresponding, in *P.*

sinuosa leaves, to 15 to 20 cm sections and, in *A. antarctica* stems, to the bottom 25 cm and two 10 cm sections above), and were blotted dry before freezing in liquid nitrogen within 15 min of collection.

Brown algal collections. Some samples for initial trials were transferred to the laboratory and stored in seawater tanks. Short incubations (<1 h) in tanks following initial collections resulted in a marked loss of activity (also seen in *Enteromorpha* sp. by Lartigue & Sherman 2002), and thus storage of unfrozen samples was avoided. Macroalgae were collected from the intertidal or subtidal region, blotted dry and frozen in liquid nitrogen within 15 min of collection. *Durvillaea potatorum* (Labillardière) Areschoug, *Macrocystis angustifolia* Bory, *Ecklonia radiata* (C. Agardh) J. Agardh, *Hormosira banksii* (Turner) Decaisne, *Cystophora torulosa* R. Brown *ex* Turner and *Sargassum* sp. were collected from Ocean Reef, near Sorrento, Australia (38° 28′ S, 144° 43′ E), in March 2000. *Fucus serratus* L., *F. vesiculosus* (L.) Lamour, *F. spiralis* L. and *Laminaria digitata* (Huds.) Lamour were collected from the intertidal region of Strangford Lough, at Portaferry, Northern Ireland (54° 23′ N, 5° 34′ W), between November 2000 and February 2001. Several macroalgal species (listed in 'Nitrate reductase activity' below) were also collected from reefs and seagrass substrates at Point Peron (32° 20′ S, 115° 42′ E) and Mettam's Pool (31° 52′ S, 115° 45′ E), near Perth, Australia, in February 2001.

Inorganic nitrogen and light profiles in seagrass meadows. Water was collected from sampling sites to characterise the vertical distribution of dissolved ammonium and nitrate availability within the seagrass meadows. Samples were collected manually using syringes attached by 1.5 m lengths of tubing to a cord, which was maintained vertical by a weight at the bottom and a float at the top. The tubing allowed water to be collected by the syringes distant from the vertical profile. The set-up was left in place for several minutes to minimise turbulence prior to sampling. Additional manual samples were taken using a syringe at the sediment surface (<1.5 cm). Duplicate profiles were sampled within each meadow at least 15 m apart and within 10 min of each other. Duplicate light attenuation profiles within *Posidonia sinuosa* and *Amphibolis antarctica* meadows were measured using a 2 pi quantum photometer (Li-Cor) attached to the top of a metal stake. PAR measurements were recorded at 50 mm intervals through the canopy by driving the stake into the sediment. To control for fluctuations in solar irradiance during the profiling period, PAR measurements were integrated over 1 min at each measuring point and all measurements were taken around midday on a cloudless day.

Nitrate reductase activity. NR activity was measured using the *in vitro* assay described by Hurd et al. (1995), modified and optimised for *Fucus serratus*, *F. spiralis* and *F. vesiculosus*. NR was extracted using a homogenisation buffer, which prevents NR degradation or oxidation, and assayed with saturating concentrations of NADH (0.2 mM) and NO_3^- (10 mM). The production of NO_2^- (resulting from NO_3^- reduction) was monitored in replicate samples, by stopping the reaction with Zn acetate at given times, and measuring NO_2^- using a standard spectrophotometric method. Hurd et al. (1995) determined that the addition of EDTA, bovine serum albumin (BSA), DL-dithiothreitol (DTT), polyvinylpyrolidone (PVP) and the detergent Triton X-100 (all Sigma) to the phosphate homogenising buffer helped maintain maximal NR activity. The effect of higher concentrations of Triton X-100 in the homogenisation medium on NR activity was tested in *F. vesiculosus* and a range of epiphytic algal species. Frozen macroalgal epiphytes on seagrasses were removed with a razor blade and ground in liquid N_2 and homogenised (0.1 g tissue with 1 ml cold extraction buffer), using a glass tube and Teflon pestle. For 2 species, this homogenising method was compared with using a commercial homogeniser (Ultra-turrax, Ika Works). The effects of PVP and the inclusion of flavin adenine dinucleotide (FAD disodium salt, Sigma) in the assay were tested for 5 macroalgae, representative of epiphyte taxa present on the seagrasses: *Dictyota* sp., (Phaeophyta), *Hypnea* sp. (Rhodophyta), *Corallina* sp. (Rhodophyta), and *Enteromorpha* sp. (Chlorophyta). The effect of BSA (Fraction V Sigma) in the homogenisation medium was tested on *Laurencia majuscula* (Harvey) Lucas (Rhodophyta) and *Dictyota* sp. NR activity was also measured in *Colpomenia peregrina* (Sauvageau) Hamel (Phaeophyta). The final buffer composition used for homogenising epiphytic algae in determination of NR profiles was 200 mM sodium-phosphate buffer, pH 7.9, with 0.3% (w/v) PVP, 2 mM DTT, 5 mM Na_2-EDTA, 3% (w/v) BSA, and 1% (v/v) Triton X-100 (all Sigma). The assay was carried out in 200 mM Na-PO_4 buffer, pH 7.9, with 0.2 mM NADH (β form, Sigma), 0.02 mM FAD (Sigma), 20% assay volume as algal extract and 10 mM KNO_3 added to start the reaction at ambient temperature: 22°C for Western Australian algae, corresponding to March water temperature in Perth coastal waters, 13°C for Irish seaweeds, or 16°C for SE Australian seaweeds (which were also assayed without FAD). The reaction was terminated with 1 M zinc acetate at time intervals 0 to 15 or 0 to 60 and the concentration of NO_2^- formed was measured as described below. NR activity was calculated using linear regression of increasing NO_2^- concentration over time. Extracts of *Dictyota* sp., *Corallina* sp., *Entermorpha* sp., *Laurencia majuscula*, and *Champia zostericola* (Harvey) Reedman & Womersley (Rhodophyta) were individually assayed and compared with an assay where extracts of these

algae were mixed in equal proportions and assayed as a pooled sample.

Analysis of ammonium, nitrate and nitrite. For the NR assays, nitrite was analysed spectrophotometrically according to Parsons et al. (1984). Nitrate plus nitrite in seawater samples was analysed in duplicate using an autoanalyser (Technicon). Ammonium concentration was estimated in duplicate samples from each sample using the fluorometric method of Holmes et al. (1999) with a Turner Designs 10AU fluorometer (Turner Systems).

Biomass estimation and total meadow nitrate assimilation. Epiphytic algal biomass on 10 stems of *Amphibolis antarctica* and 10 leaves of *Posidonia sinuosa* from the Shoalwater Bay sites was removed with a razor blade, described by Kendrick & Lavery (2001), and frozen in liquid N_2 for determination of frozen mass. The samples were subsequently thawed, ovendried at 60°C and the relationship between frozen and dry mass determined by regression, separately for *A. antarctica* and *P. sinuosa* epiphyte assemblages. These relationships were used to convert NR activity from a frozen to a dry mass basis, and to estimate total NR activity in epiphyte biomass represented on each portion of leaf or stem.

The NO_3^- assimilation capacity represented by biomass-specific NR activity levels in macroalgal epiphytes was converted to an areal basis using the measurements of epiphyte biomass per leaf or stem (above) and published estimates of shoot density (a monospecific *Posidonia sinuosa* meadow from Shoalwater Bay of 956 shoots m^{-2} [Cambridge & Hocking 1997] and a median shoot density of 750 shoots m^{-2} from a range reported for *Amphibolis* spp. meadows in Perth coastal waters [Kendrick et al. 1998]).

NO_3^- assimilation can be defined as the reduction of NO_3^- into amino acids, whereas N incorporation involves reduction of inorganic N to macromolecules including proteins. Estimates of potential NO_3^- assimilation rates for the macroalgae were compared with estimates of N incorporated into above-ground new growth by seagrasses, excluding epiphytes, calculated from published values of 25.6 mg N m^{-2} d^{-1} for *Posidonia australis* and 49.7 mg N m^{-2} d^{-1} for *Amphibolis antarctica* in Shark Bay (Walker & McComb 1988). To conservatively account for the discrepancy between N assimilation and N incorporation, the lowest incorporation efficiency of 16% in the seagrass *Thalassia testudinum* reported by Lee & Dunton (1999) was used to convert N incorporation to N assimilation rates for the seagrass component.

Data analysis. Differences in NR activity between different leaf or stem portions were evaluated separately for each seagrass using a 1-way ANOVA with a Tukey-Kramer pairwise post-hoc analysis testing for significance at $p < 0.05$ (SigmaStat, SPSS). NR activity in epiphytes from the 2 seagrass meadows were compared with a 2-way ANOVA (SigmaStat). Light attenuation between the meadows was compared using a paired *t*-test (SigmaStat).

RESULTS

Optimisation of nitrate reductase assay

Application of the optimised *in vitro* NR assay yielded NR activity in all macroalgal species tested. Assays of NR activity in *Fucus* species showed that increasing the concentration of Triton X-100 from 0.1%, used by Hurd et al. (1995), to 1% dramatically increased the NR activity extracted (results from present study [see Fig. 1A, Table 1] and from previous studies [see Table 1]). Increasing the Triton X-100 concentration from 1 to 2% did not yield higher NR activity from any of the test species (Fig. 1A, other species not shown, n = 3). The NR activity in *Fucus* species from Ireland was the highest, exceeding previous esti-

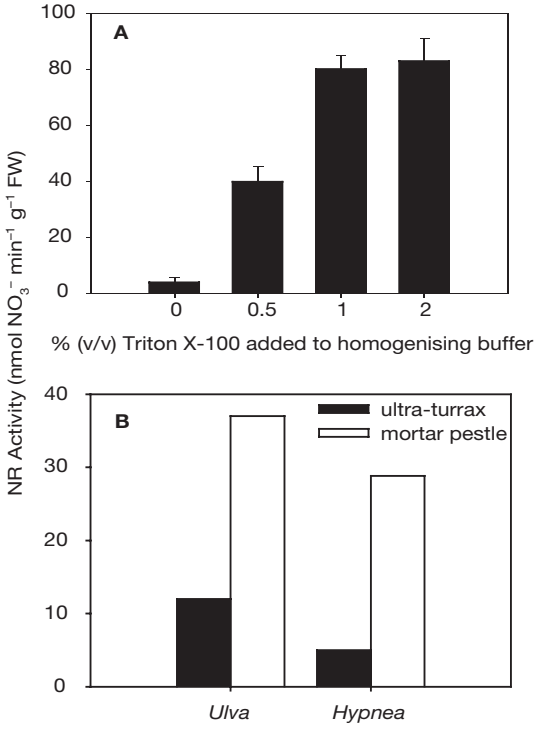

Fig. 1. Effect of homogenising conditions on nitrate reductase (NR) activity measured in macroalgal extracts. (A) Effect of different concentrations of Triton X-100 in the homogenising buffer on NR activity measured in *Fucus vesiculosus*. Extracts were homogenised with a mortar and pestle. Bars are mean; error bars are SD, n = 3. (B) Effect of manual glass mortar and Teflon pestle versus a commercial homogeniser on yield of NR activity in *Ulva* sp. and *Hypnea* sp., n = 1. FW: frozen thallus weight

mates nearly 10-fold (Table 1). In epiphytic algae from Western Australia, the highest NR activities were measured in *Ulva* and *Hypnea* species, at approximately 50 nmol NO_3^- min^{-1} g^{-1} frozen thallus weight (FW), and a minimum activity in any extract of 2 nmol NO_3^- min^{-1} g^{-1} FW. The method of homogenisation was important in the yield of NR activity extracted from thalli. A 3-fold higher activity was measured in *Ulva* and *Hypnea* species when using a glass tube and mechanically driven close-fitting Teflon pestle compared to extracts made using an Ultra-Turrax tissue homogeniser, even when ground with identical buffer (Fig. 1B). The NR activity yield was not affected by inclusion or exclusion of 0.3% insoluble PVP. Although the inclusion of FAD

Table 1. Comparison of maximum nitrate reductase (NR) activities measured in extracts of macroalgae from a range of taxonomic groups, using the *in vitro* method and normalised to frozen weight (FW). Where included, measures of variation are standard deviation unless indicated otherwise (SE = standard error). W Aust: Perth coastal waters, Western Australia; SE Aust: Sorrento, Victoria; Ireland: Strangford Lough, N. Ireland. FAD: flavin adenine dinucleotide

Species (sampling site or laboratory cultured)	Max. NR activity (nmol NO_3^- min^{-1} g^{-1} FW)	Notes/ comments	Source
Phaeophyta			
Macrocystis integrifolia (BC, Canada)	9.61 (±1.8)		Hurd et al. (1995)
Macrocystis angustifolia (South Africa)	3.3		Haxen & Lewis (1981)
Macrocystis angustifolia (Cultured)	11.7	[a](1.8 mM)	Haxen & Lewis (1981)
Laminaria digitata (Scotland)	5.3		Davison & Stewart (1984a)
Laminaria digitata (Ireland)	60 (±5.4)	+FAD	Present study
Fucus gardneri (BC, Canada)	35.99 (±1.58)		Hurd et al. (1995)
Fucus serratus (Ireland)	165 (±18)	+FAD	Present study
Fucus spiralis (Ireland)	273 (±8)	–FAD	Present study
Fucus vesiculosus (Ireland)	290 (±15)	–FAD	Present study
Pelvetia canaliculata (Ireland)	122 (±9.8)	–FAD	Present study
Dictyota sp. (W Aust)	40.6 (±1.6)	+FAD	Present study
Sargassum sp. (W Aust)	10.8	+FAD	Present study
Sargassum sp. (SE Aust)	31.9	[b]	Present study
Ecklonia radiata (SE Aust)	16.7	[b]	Present study
Durvillaea potatorum (SE Aust)	4.5	[b]	Present study
Cystophora torulosa (SE Aust)	4.5	[b]	Present study
Hormosira banksii (SE Aust)	1.8	[b]	Present study
Macrocystis angustifolia (SE Aust)	5.5	[b]	Present study
Colpomenia peregrina (W Aust)	2.5		Present study
Rhodophyta			
Porphyra yezoensis (Japan)	33.2		Araki et al. (1979)
Porphyra perforata (BC, Canada)	20.8	[c]	Thomas & Harrison (1988)
Porphyra sp. (BC, Canada)	4.0 (±0.96)		Hurd et al. (1995)
Corallina vancouvensis (BC, Canada)	22.6 (±1.31)		Hurd et al. (1995)
Corallina sp. (W Aust)	30.8 (±2.1)		Present study
Laurencia majuscula (W Aust)	3.4 (±1.6)		Present study
Chondrous crispus (Ireland)	108 (±17)		Present study
Palmaria palmata (Ireland)	9.7 (±3.7)		Present study
Hypnea sp. (W Aust)	48.7 (±1.9)		Present study
Champia sp. (W Aust)	17.1 (±1.3)		Present study
Gracilaria tenuisipitata (Cultured)	43.3	[a] (500 µM)	Lopes et al. (1997)
Gelidium sp. (AL, USA)	11.50 (±0.33 SE)		Lartigue & Sherman (2002)
Chlorophyta			
Ulva sp. (AL, USA)	3.67 (±0.17 SE)		Lartigue & Sherman (2002)
Ulva sp. (BC, Canada)	9.3 (±1.6)		Hurd et al. (1995)
Ulva sp. (Ireland)	45.9 (±14.2)		Present study
Ulva sp. (W Aust)	37.3 (±5.4)		Present study
Enteromorpha sp. (AL, USA)	9.67 (±1.17 SE)		Lartigue & Sherman 2002)
Enteromorpha (BC, Canada)	26.7		Hurd et al. (1995)
Acetabularia mediterranea (Cultured)	120	[c]	Balandin & Aparicio (1992)

Key to localities in this study:

[a] NO_3^- enriched preincubation conditions (NO_3^- concentration in parentheses)

[b] Assayed without FAD; effect of FAD not tested

[c] NR activity per protein converted to per mass using a value of 10 mg protein g^{-1} wet weight from *A. mediterranea*, or 25 mg protein g^{-1} wet weight used by Hurd et al. (1995) for *Pophyra perforata*

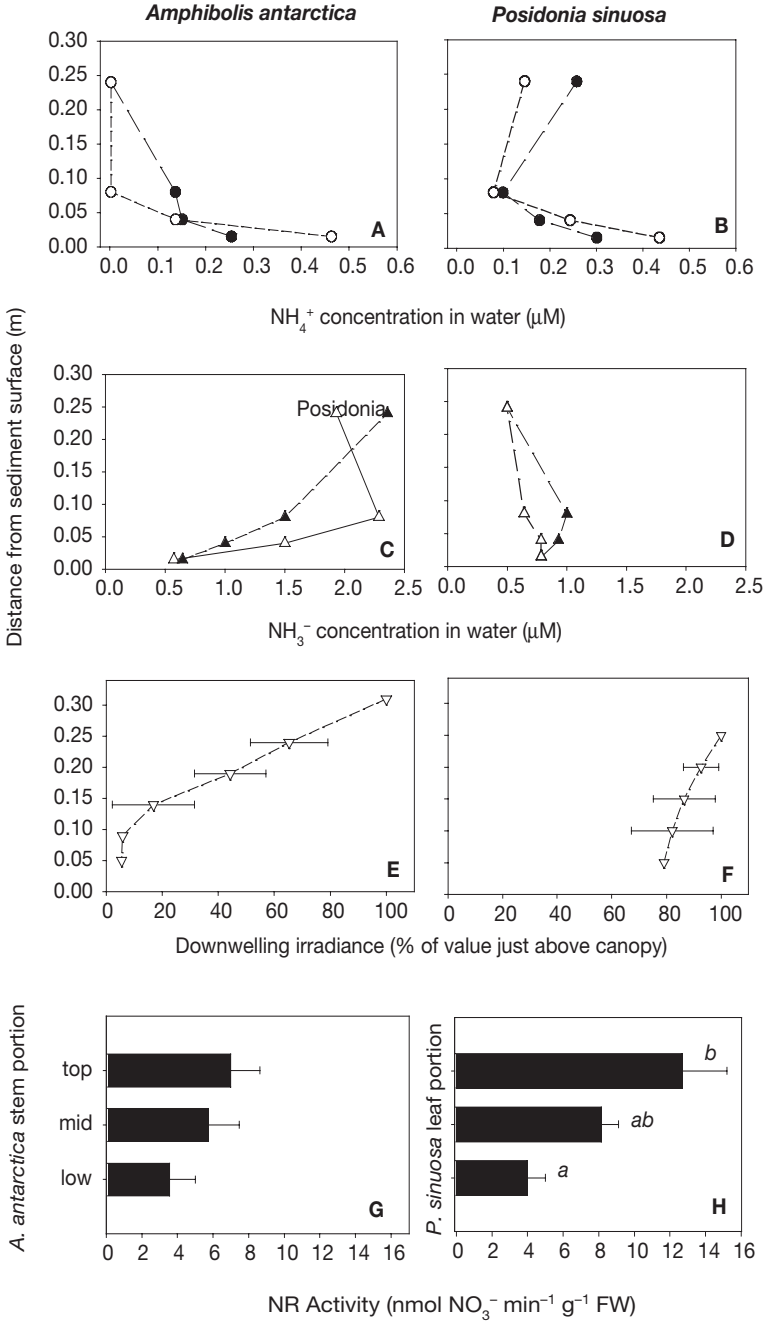

Fig. 2. Dissolved inorganic nitrogen concentrations, irradiance and NR activity in epiphytic algae over a vertical gradient from the sediment surface in (A,C,E,G) *Amphibolis antarctica* and (B,D,F,H) *Posidonia sinuosa* meadows in Shoalwater Bay, Western Australia. (A–D) Vertical profiles through the water column and canopy of (A,B) NH_4^+ and (C,D) NO_3^- + NO_2^- concentration. Plots show duplicate profiles sampled through each meadow type. (E,F) Light attenuation through the canopy of *A. antarctica* and *P. sinuosa* meadows (mean ± SD, n = 2). (G–H) Vertical distribution of mean NR activity (+1 SD) in algal epiphytes from *A. antarctica* and *P. sinuosa* meadows, n = 6 to 14. low: epiphytes removed from the seagrass stem or leaf closest to the sediment surface; mid: epiphytes removed from middle part of seagrass stem or leaf; top: epiphytes removed from portion of seagrass furthest from the sediment (see text for details). Bars with statistically different values are labelled with different letters

in the assay improved activity in *Fucus serratus* and *Laminaria digitata*, it did not significantly elevate activity in *F. spiralis*, *F. vesiculosus*, and the epiphytic species *Corallina* sp., *Hypnea* sp., *Dictyota* sp. or *Enteromorpha* sp., although NR activity was highly variable among replicate plants of *Dictyota* sp. and *Enteromorpha* sp. (n = 3 to 6). The exclusion of BSA from the homogenisation buffer resulted in a 25 to 40% decline in measured NR activity in extracts of *F. vesiculosus*, *Laurencia majuscula* and *Dictyota* sp. (n = 3; not shown). In a combined assay, there was only a very minor inhibition of NR activity in each species by the inclusion of extracts of other species; individually assayed extracts of the epiphytic algae yielded a mean NR activity of 2.86 nmol NO_3^- min^{-1} g^{-1} FW and in the pooled sample, the NR activity was 2.65 nmol NO_3^- min^{-1} g^{-1} FW, within 7% of the mean value.

Nutrient and light profiles in seagrass meadows

Dissolved inorganic nitrogen concentrations were low in the open coastal water sampled in replicate vertical profiles through seagrass meadows in Shoalwater Bay (Fig. 2A–D). In both *Amphibolis antarctica* and *Posidonia sinuosa* meadows, the trend was for a higher NH_4^+ concentration in the water close to the sediment surface (Fig. 2A,B). There was an increase in NO_3^- concentration with distance from the sediment in the *A. antarctica* meadows but no statistically significant vertical variation in NO_3^- concentration in *P. sinuosa* meadows (Fig. 2C,D). The lowest NH_4^+ concentration within the seagrass canopy and in the overlying water column was observed in the *A. antarctica* meadows. The concentration of NH_4^+ and NO_3^- at the sediment surface was similar (0.3 to 0.5 µM for NH_4^+ and 0.6 to 0.8 µM for NO_3^-) in both seagrass types. In the overlying water column, NO_3^- concentrations were higher in *A. antarctica* than in *P. sinuosa* meadows, but the NH_4^+ concentrations were more similar (<0.5 µM) above both meadow types.

The attenuation of PAR through the canopy was significantly greater in the *Amphibolis antarctica* meadow than in the *Posidonia sinuosa* meadow (<0.001) (Fig. 2E,F). Mean canopy attenuation coefficients were 10.9 ± 3.7 and 1.3 ± 1.1 m^{-1} (n = 2), respectively, for *A. antarctica* and *P. sinuosa* canopies.

Vertical distribution of NR activity

NR activity measured in algal epiphytes from seagrass leaves increased with distance from the sediment surface in *Posidonia sinuosa* meadows (Fig. 2H; $p = 0.0283$, $F = 4.053$, df 2,28; 1-way ANOVA). Pairwise analysis suggested that the differences in NR activity in epiphytes from the lowest and uppermost portions of leaves of *P. sinuosa* were significant ($p < 0.05$), but differences in NR activity of algae from the top and middle or lowest and middle portions of the leaves were not significant (Fig. 2H). While a similar trend was apparent for *Amphibolis antarctica* stems, this was not statistically significant ($p > 0.5$, Fig. 2G). NR activity in epiphytes sampled from *P. sinuosa* leaves was higher than the activity observed in epiphytes from *A. antarctica* stems ($p < 0.008$, 2-way ANOVA). When plotted against the vertical distribution of inorganic N (Fig. 3), the NR activity in epiphytic algae on *A. antarctica* showed a close negative correlation with NH_4^+ ($r^2 = 0.999$; NR activity = -9.757 $[NH_4^+] + 7.056$) and positive correlation with NO_3^- concentration ($r^2 = 0.991$). The relationship between inorganic N concentration and NR activity in *P. sinuosa* epiphytes was less clear-cut; there was a fair correlation and negative relationship between NR activity and NH_4^+ concentration ($r^2 = 0.792$; NR activity = $-34.156 \times [NH_4^+] + 17.421$) but no relationship to NO_3^- concentration in the water column ($r^2 = 0.199$). The *x*-intercepts for the linear relationship between NR activity and NH_4^+ concentration (Fig. 3A) suggested that the ambient NH_4^+ concentration required to achieve no NR activity was 1.38 µM (*A. antarctica* epiphytes) and 1.96 µM (*P. sinuosa* epiphytes).

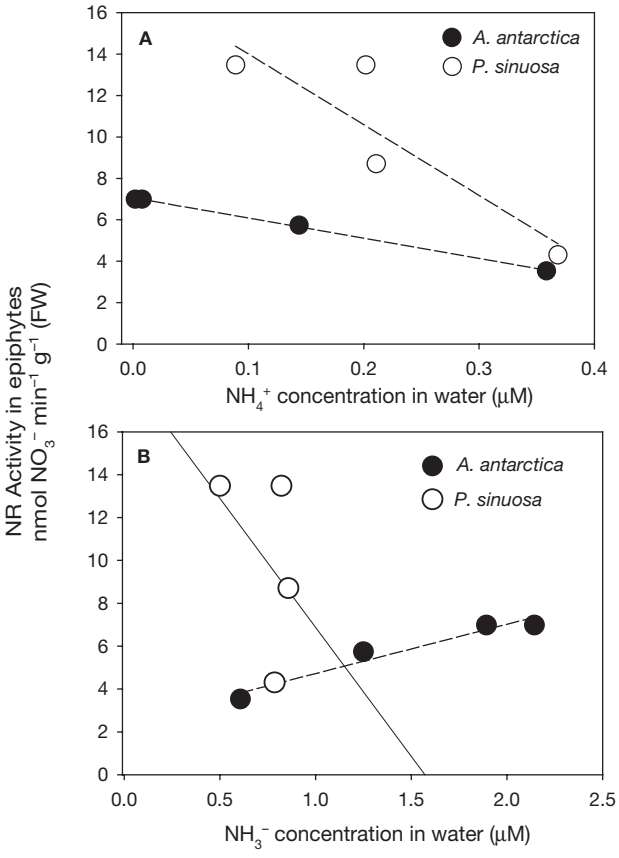

Fig. 3. NR activity in epiphytes on seagrasses plotted against (A) ambient ammonium and (B) nitrate concentrations in the water column. Lines are linear regressions (Sigmaplot): $r^2 = 0.9986$ and 0.9906 for *Amphibolis antarctica* and $r^2 = 0.7922$ and 0.1994 for *Posidonia sinuosa* for NH_4^+ and NO_3^-, respectively

Distribution of epiphyte biomass and NR activity

Maximum NR activity was used to derive the estimated NO_3^- assimilating capacity associated with macroalgal epiphyte assemblages. Even though the biomass-specific NR activity in *Posidonia sinuosa* epiphytes was higher (Fig. 2G,H), each *Amphibolis antarctica* stem supported twice the nitrate assimilation capacity of each *P. sinuosa* leaf because of the higher epiphytic macroalgal biomass (Table 2). The middle stem section of *A. antarctica* often supported large clumps of a single alga, evident in the vertical biomass distribution shown in Table 2. On *P. sinuosa* leaves, the greatest algal biomass accumulation was on the oldest, uppermost section of the leaf.

Using published estimates of shoot or leaf areal density (Cambridge & Hocking 1997, Kendrick et al. 1998), the calculated area-specific NO_3^- assimilation capacity of *Amphibolis antarctica* epiphyte assemblages was estimated at almost double that of the epiphytes in *Posidonia sinuosa* meadows (Table 2). In both seagrass types, the N assimilation capacity of the macroalgal epiphytes was more than double the estimated maximum rate of N acquisition by the seagrass component of the meadow.

DISCUSSION

Nitrate reductase assay optimisation

NR activity could be measured in all 22 species of algae from Ireland, SE Australia and Western Australia (Table 1). The optimised *in vitro* NR activity assay can be applied to diverse red, green and brown macroalgal taxa, despite previous reports indicating poor or no yield of NR activity in some species using an *in vitro* assay (Thomas & Harrison 1988, Corzo & Niell 1991). In *Fucus* and *Laminaria* species, the NR activity mea-

Table 2. Algal biomass epiphytic on stems of *Amphibolis antarctica* and leaves of *Posidonia sinuosa* collected in Shoalwater Bay, and the vertical distribution of NO_3^- assimilation capacity (standard error [SE], n = 6 to 14). Assimilation is conversion of NO_3^- to NH_4^+ or amino acids (based on NR activity estimates). Biomass estimates are means of 20 leaves or stems (SE). NO_3^- assimilation capacity for meadows were estimated using published values of shoot density. No co-efficient of variation available for the shoot density estimates. Top: portion of seagrass furthest from the sediment; Mid: portion of seagrass in middle; Low: the seagrass stem or leaf closest to the sediment surface

	Epiphyte dry biomass (g leaf^{-1} or stem^{-1})	NO_3^- assimilation capacity in epiphytes (µmol NO_3^- leaf^{-1} or stem^{-1} d^{-1})	Epiphyte NO_3^- assimilation capacity in meadow (mmol NO_3^- m^{-2} d^{-1}	Estimated seagrass N incorporation (mmol N m^{-2} d^{-1})	Estimated maximum seagrass N assimilation (mmol N m^{-2} d^{-1})
Posidonia sinuosa				1.83 (0.14)[c]	10.99 (0.84)[d]
Top	0.172 (0.044)	22.24 (4.24)			
Mid	0.028 (0.004)	2.31 (1.51)			
Low	0.026 (0.003)	1.08 (1.58)			
Total per leaf	0.225 (0.044)	25.62 (4.24)	24.65[a]		
Amphibolis antarctica				3.5 (1.78)[c]	21.3 (10.7)[d]
Top		0.78 (0.11)	27.63 (2.65)		
Mid	1.46 (0.34)	32.79 (3.85)			
Low	0.053 (0.013)	0.91 (2.15)			
Total per stem	2.30 (0.34)	61.33 (3.85)	45.99[b]		

[a]Using a mean shoot density, 956 shoots m^{-2}, reported for *P. sinuosa* meadow, Shoalwater Bay (Cambridge & Hocking 1997)

[b]Using a median shoot density of 750 shoots m^{-2} from a range reported for *Amphibolis* meadows in Perth coastal waters (Kendrick et al. 1998)

[c]Estimates of N incorporated into above-ground new growth by seagrasses (inorganic to organic N), excluding epiphytes, calculated from average yearly values of 25.6 ± 2 mg N m^{-2} d^{-1} for *Posidonia australis* and 49.7 ± 25 mg N m^{-2} d^{-1} for *A. antarctica* in Shark Bay (Walker & McComb 1988)

[d]Using the minimum efficiency of N incorporation of 16% of the inorganic N taken up by seagrass (*Thalassia testudinium*; Lee & Dunton 1999), the N incorporation rates from Walker & McComb (1988) were used to estimate a maximum N assimilation rate by *P. sinuosa* and *A. antarctica*

sured in this study was much higher than in previous reports (Table 1), probably reflecting the effect of higher Triton X-100 concentration and an optimised assay. For other species, the rates of NR activity measured in this study are comparable with those of previous studies (Table 1), e.g. *Corallina vancouvensis* from British Columbia (Hurd et al. 1995) and *Corallina* sp. from Australian seagrass stems yielded an NR activity of 22.6 ± 1.31 and 30.8 ± 2.1 nmol NO_3^- min^{-1} g^{-1} FW, respectively.

The homogenisation method was important in extracting NR activity from the algae, and the combination of liquid N_2 grinding (Hurd et al. 1995, Lopes et al. 1997, Lartigue & Sherman 2002) and the Teflon pestle and glass tube homogeniser is recommended over the automated tissue homogenisers (e.g. Davison & Stewart 1984a). Hurd et al. (1995) also noted that sonication was not recommended. Extraction efficiency also improved with 1% Triton X-100, rather than the 0.1% used by Hurd et al. (1995). The present study confirms the importance of including DTT and BSA in the extraction buffer to protect from enzyme inactivation and proteolytic digestion although Lartigue & Sherman (2002) found casein more effective than BSA in stablising NR activity in *Enteromorpha* sp. FAD and PVP seem to be less important in optimising NR activity (see Everest et al. 1984, Hurd et al. 1995, Lopes et al. 1997) and the requirement for these additions may be species-specific.

The lack of inhibition of net NR activity in combined extracts of different epiphytic seaweeds indicates that the assay technique can be applied with confidence to epiphytic algal assemblages. Despite the mixed samples, the range of NR activities in the epiphytic algae from seagrass leaf or stem portions (4 to 15 nmol NO_3^- min^{-1} g^{-1} FW) is comparable with values in individual samples of red, brown and green algal species (Table 1; Hurd et al. 1995).

Vertical profiles of NR activity

NR activity of macroalgal epiphytes on *Amphibolis antarctica* and *Posidonia sinuosa* was inversely correlated with NH_4^+ concentration in the seawater (which decreased with distance from the sediment surface). This supports the hypothesis that NH_4^+ released from the sediments suppresses expression of NR activity in macroalgal epiphytes on *P. sinuosa* seagrass. Dissolved NH_4^+ released from the sediments may be preferentially taken up by macroalgae within seagrass meadows (Thomas & Harrison 1985), and uptake of NH_4^+

can suppress the expression of NR in algae (Berges et al. 1995). The relationship between NO_3^- and NH_4^+ availability and NR expression in algae has been more comprehensively characterised in phytoplankton and is less well understood in macroalgae. However, suppression or inactivation of NR activity by the presence of NH_4^+ is evident in Fig. 3A and has been shown in red macroalgae (Thomas & Harrison 1985, Ganesan et al. 2001), in the brown alga *Laminaria digitata* (E. B. Young unpubl.), and in the green alga *Acetabularia mediterranea* (Balandin & Aparicio 1992). If NH_4^+ availability suppresses NR in epiphytic algae, NH_4^+ diffusion from the sediments into the water column may result in the reduced NR activity levels observed in *P. sinuosa* epiphytes growing closer to the sediment surface. Extrapolation of the relationship between NR activity in macroalgal epiphytes and NH_4^+ concentration (Fig. 3A) suggests that NR activity would be completely inhibited at water column concentrations between 1 and 2 µM. These values are close to that estimated by Dortch (1990) for complete inhibition of NR activity in phytoplankton.

The vertical profile in epiphytic macroalgal NR activity in *Amphibolis antarctica* was similar to that in *Posidonia sinuosa* but was not statistically significant. Greater replication may have reduced the variance in our data and possibly resulted in a significant trend in both seagrass types. In addition to a strong NH_4^+ gradient through the *A. antarctica*, there was an opposing vertical profile in NO_3^- (compare Fig. 2C and D, Fig. 3B) both of which are expected to influence the expression of NR activity in algae; NR expression requires the presence of NO_3^- (Solomonson & Barber 1990) and there was a weak positive linear relationship between NR activity and NO_3^- concentration in *A. antarctica* epiphytes (r^2 = 0.991; Fig. 3B), although no clear-cut relationship was observed for *P. sinuosa* epiphytes (Fig. 3B). In phytoplankton, NR activity can be enhanced by the presence of low NO_3^- concentration, probably to ensure efficient assimilation of what NO_3^- is available (Dortch 1990, Berges et al. 1995); recurrent or continual low NO_3^- concentration in Perth coastal waters (Johannes et al. 1994) may thus stimulate NR activity in seagrass epiphytes.

Despite the apparent similarity in the vertical trend in epiphyte NR activity through the canopies of both seagrass species, it is possible that the lack of statistical significance for the data from *Amphibolis antarctica* meadows reflects a real difference between the 2 seagrass communities. This could be due to several factors including NO_3^- and NH_4^+ availability, light and epiphyte composition. It is unknown if the differences observed in water column NO_3^- concentrations between the 2 meadow types endure long enough to affect algal physiology and the extent to which inorganic N concentrations in seagrass meadows are seasonally or diurnally persistent, although this warrants further examination. Rapid turnover of NH_4^+ (and urea) excreted by epifauna on the seagrass host could also contribute to NH_4^+ rather than NO_3^- uptake by algae and seagrasses, but this may not be evident from water column sampling. Light is an important environmental factor regulating NR activity in algae, stimulating both NO_3^- uptake and expression of NR in macroalgae (Davison & Stewart 1984b, Gao et al. 1992, Lopes et al. 1997). The branching habit and high epiphyte loads resulted in the relatively high attenuation of light observed in *A. antarctica* meadows (see also Brush & Nixon 2002). However, the lack of a steep irradiance gradient in the *Posidonia sinuosa* meadow, where significant vertical variation in NR activity was observed, suggests that irradiance was not a major determinant of the observed trends in NR activity in seagrass epiphytes. The irradiance measured close to the sediment in *A. antarctica* and *P. sinuosa* were 40 and 800 µmol photons m^{-2} s^{-1}, respectively, neither of which represents growth-limiting values for most macroalgae (Dring 1982).

NR activity may also be related to differences between the 2 seagrasses in the vertical patterns of epiphyte composition and turnover rate of leaves and stems to which the epiphytes attach. For seagrasses with strap-like leaves, such as *Posidonia sinuosa*, the diversity, as well as biomass, of epiphytic algae is highest on the leaf apex (Trautman & Borowitzka 1999). *P. sinuosa* leaves have an average longevity of 245 d (Marbà & Walker 1999) and grow from the base, with the oldest part of the leaf at the top of the canopy where the greatest epiphyte biomass was observed (Table 2; Trautman & Borowitzka 1999). This 'conveyer belt' of *P. sinuosa* leaf growth means that algae recruited to the younger leaf surface that remain attached to the leaf will be more mature when they reach the top of the leaf, a factor which may influence NO_3^- metabolism. This spatial pattern in maturity may contribute to a pronounced gradient of NR activity in epiphytes on *P. sinuosa. Amphibolis* species do not show the same spatial relationship with epiphyte recruitment as the strap-leafed species (Lavery & Vanderklift 2002). Macroalgal biomass on *A. antarctica* was mostly found attached to the perennial stems, located in the middle section of the canopy (Table 2). This could eliminate any vertical gradient in NR activity amongst algal epiphytes related to age of algal epiphytes.

Leaving aside any possible differences in the vertical distribution of NR activity through the canopies, the NR rates on *Posidonia sinuosa* were clearly higher than those on *Amphibolis antarctica*. The current study did not include detailed analyses of species composition

and there are few comparative studies of the epiphyte assemblages on different seagrasses. However, previous work has shown a significant variation in the composition of epiphytes among seagrass species (Trautman & Borowitzka 1999, Lavery & Vanderklift 2002). It is possible, therefore, that some of the differences in mass-specific NR activity between *A. antarctica* and *P. sinuosa* epiphytes were due to differences in the species composition.

Ecosystem-level scaling

Potential rates of NO_3^- assimilation, estimated from observed NR activity, demonstrated the considerable capacity of macroalgae in seagrass meadows to capture NO_3^- from the water column. Recent studies (e.g. Lee & Dunton 1999) attempted to estimate annual N budgets for seagrasses using measured inorganic N uptake rates. Using NR activity, the NO_3^- assimilating capacity of epiphytic algae was estimated to be more than double the N incorporation rates of seagrasses in both *Amphibolis antarctica* and *Posidonia sinuosa* meadows (Table 2). However, this may significantly overestimate the relative assimilation rates of epiphytic macroalgae, for 2 reasons. First, N incorporation estimates for the seagrasses were based on growth rates, and will underestimate the rate of N assimilation within seagrass tissues. We did correct for this using a published incorporation rate from the seagrass *Thalassia testudinum*, for which inorganic N uptake was found to be up to 6 times greater than N incorporation into new growth; this was attributed to excretion of dissolved inorganic and organic N (Lee & Dunton 1999). Second, the epiphyte biomass recorded at Shoalwater Bay was high compared to other seagrass meadows in the region, principally as they are summer values, and seasonal temperature fluctuations may influence assimilation rates. While shoot densities remain similar in both species over an annual cycle (Cambridge & Hocking 1997, Lavery et al. 1998), epiphyte loads vary significantly over a year. At Shoalwater Bay, in summer, the epiphyte load was approximately 0.225 g dry weight per *P. sinuosa* leaf and 2.3 g dry weight per *A. antarctica* stem. Over an annual cycle, Cambridge & Hocking (1997) measured annual epiphyte biomass means of approximately 0.024 g shoot^{-1} for *P. sinuosa*, and Lavery et al. (1998) estimated an annual average of 0.913 g shoot^{-1} for *Amphibolis griffithii*, approximately 10 and 40% of those measured in February at Shoalwater Bay, respectively. This suggests that our estimates based on Shoalwater Bay data from summer are likely to be at the upper end of potential NO_3^- assimilation, and that annual averages could be about 1 order of magnitude lower than those we have estimated.

Allowing for these discrepancies, the NO_3^- assimilating capacity in macroalgal epiphytes was at least comparable to the estimated rate of N acquisition by *Posidonia sinuosa* or *Amphibolis antarctica* (Table 2), so epiphytic algae on these seagrasses represent a potentially major reservoir for water column NO_3^-. However, given the low N concentration in water around the meadows, and that NR is the rate-limiting enzyme in NO_3^- assimilation, N incorporation may be limited by availability of NO_3^- rather than by NO_3^- assimilation capacity. The potential contribution of epiphytes to total N assimilation in a seagrass meadow could be higher if the epiphytes assimilate significant amounts of NH_4^+ from the water column. NH_4^+ concentration in Perth coastal waters is very low, and NO_3^- is the dominant form of dissolved inorganic N (Johannes et al. 1994; Fig. 2) though epifaunal excretion may supply additional NH_4^+ for uptake.

There are few estimates of NR activity from seagrasses, but Touchette & Burkholder (2001) recently measured NR activity in *Zostera marina* using an *in situ* assay, and reported 8.3 nmol NO_3^- min^{-1} g^{-1} tissue under ambient NO_3^- conditions. This is similar to the range of values observed for macroalgal epiphytes from *Posidonia sinuosa* and *Amphibolis antarctica*, although the *in situ* and *in vitro* assays may not be strictly comparable. The comparison of N incorporation by epiphytes versus seagrasses has not accounted for below-ground N acquisition by seagrasses, although estimates of this vary from half the total N acquisition in *Thalassia testudinum* (Lee & Dunton 1999), to somewhat less in *A. antarctica* (Pedersen et al. 1997) and minimal in *P. oceanica* (Kraemer et al. 1997). However, the N incorporation values (Walker & McComb 1988) we used were for above-ground biomass only. Furthermore, production rates of algal epiphytes are higher than those of seagrasses (Cambridge & Hocking 1997), and macroalgae can also take up inorganic N faster than seagrasses (e.g. Pedersen 1994, cf. Pedersen et al. 1997), competing effectively for dissolved inorganic N in the water column.

Based on the above estimates, epiphytes could play a significant role in nitrate acquisition in both the seagrass meadow types we examined. Under oligotrophic conditions, epiphytes may confer a significant advantage to seagrass ecosystems by assimilating N and by reducing export of N from the meadow by contributing to the trapped detrital component of meadows. Within a tropical seagrass community, living seagrass only contained 6% of total N, with over 90% total N represented in particulate detritus (Boon 1986), to which epiphytes make an important contribution. Of course any benefits conferred by macroalgal epiphytes on nutrient dynamics of seagrass meadows may be offset by a reduction of light reaching the seagrass leaves.

However, in clear oligotrophic waters at low and moderate latitudes, this reduction is unlikely to limit growth, and epiphytes may also shield seagrasses from harmful UV radiation.

SUMMARY AND CONCLUSIONS

The improved *in vitro* NR assay was applicable to a diverse range of 22 macroalgal taxa; with liquid nitrogen freezing soon after collection, the assay can be used to estimate NR activity, and thus estimate a maximum NO_3^- assimilation rate, in field samples. A decrease in the NR activity in *Posidonia sinuosa* epiphytes growing closer to the sediments, and tight negative relationships between NR activity and water column NH_4^+ concentration, support the hypothesis that higher NH_4^+ concentration close to the sediment suppresses NR activity in macroalgal epiphytes on *P. sinuosa* leaves. The lower NR activity overall and lack of significant vertical variation in NR activity in algae growing on *Amphibolis antarctica* could relate to higher NO_3^- availability or confounding effects of macroalgal species composition and distribution. The potential NO_3^- assimilation by macroalgal epiphytes on seagrasses represents a major sink for water-column NO_3^-, but NO_3^- capture and incorporation by macroalgal epiphytes may be more limited by NO_3^- availability than assimilation capacity.

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