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NOTE

^{15}N enrichment as a method of separating the isotopic signatures of seagrass and its epiphytes for food web analysis

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ABSTRACT: Stable isotope analysis of food webs is of limited use where there is little or no difference in the natural abundance isotopic ratios of potential food sources. ^{15}N-enriched potassium nitrate was used to differentially label 2 potential food sources for seagrass fauna: seagrass and its attached epiphytes. Different combinations of exposure time to the enriched substrate and different concentrations of enriched substrate were used to maximise the difference in $\delta^{15}N$ signature between the 2 food sources. After adding the enriched substrate $\delta^{15}N$ values of epiphytes ranged from 87 to 713‰, and were consistently higher than the $\delta^{15}N$ values of seagrass, which ranged from 25 to 90‰. Enriched substrate additions every 3 d resulted in the greatest sustained separation between seagrass and epiphytes over 18 d. The results demonstrate that enriched ^{15}N tracers are useful for separating the $\delta^{15}N$ signatures of previously difficult to distinguish primary sources, and that this technique has the potential to resolve ambiguous natural abundance isotope results.

KEY WORDS: Algae · Stable isotopes · ^{15}N-enriched isotope tracers · Trophic webs · *Zostera capricorni*

The past 2 decades have seen a rapid expansion in the use of natural abundance stable isotopes such as $\delta^{13}C$, $\delta^{15}N$ and, to a lesser extent, $\delta^{34}S$ to analyse aquatic food webs (e.g. Peterson et al. 1985, Sullivan & Moncreiff 1990, Bunn & Boon 1993). The advantages of this approach over techniques such as gut content analysis are that it distinguishes assimilated rather than ingested foods, and it can represent a history of assimilation over longer periods (Gearing 1991). A major limitation, however, is that potential food sources must be isotopically distinct in 1 or more of the isotopes examined (Gearing 1991). Additional problems can arise when there are more than 2 distinctive primary sources, or where a consumer's signature could have resulted from assimilating either 1 source or a mixture of 2.

One method which overcomes the limitations of this technique, as well as the low level of resolution observed in natural abundance stable isotope food web studies, is the manipulation of the isotopic ratio of a particular primary food source, by adding a ^{15}N- or ^{13}C-enriched substrate. This enriched signature can then be traced through the food web. Few studies of food webs have used enriched stable isotope tracers (e.g. Kling 1994, Hall 1995), and this technique does not appear to have been applied in estuarine or marine ecosystems.

Seagrass and epiphytic algae attached to seagrass leaves are both potential food items for animals inhabiting seagrass meadows. Many animals have been shown to ingest seagrass. McRoy & Helfferich (1980) list 154 such species including dugongs *Dugong dugon*, blue crabs *Callinectes sapidus*, numerous birds, and 59 species of fish. Seagrass, however, is generally considered to be of little nutritional importance compared to other plant sources, such as epiphytic algae (Klumpp et al. 1989). Epiphytes can be of higher nutritional quality than seagrasses and are often mechanically more accessible (Bell & Pollard 1989). In some cases, the greater importance of algae than seagrass to consumers has been supported by the $\delta^{13}C$ values of the consumers (e.g. Kitting et al. 1984), while in other studies the isotope ratios of seagrass and epiphytes could not be differentiated (Fry et al. 1982, Loneragan et al. 1997).

In applying enriched-tracer techniques to a seagrass system for the first time, we attempted to: (1) separate the isotopic signature of 2 potential food sources (seagrass and its attached epiphytes) for seagrass fauna in the short term, using a ^{15}N-enriched substrate, and

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(2) determine an enriched substrate addition regime which creates the greatest separation in $\delta^{15}N$ values of seagrass and its epiphytes over an extended period.

Materials and methods. *Zostera capricorni* is the dominant shallow-water seagrass species along the east coast of Australia and within Moreton Bay. Pieces of seagrass turf, including the substrate, the root system, and the epiphytes attached to the seagrass leaves, were removed from Toondah Harbour, in Moreton Bay, Australia (27° 38' S, 153° 25' E), using a spade. The seagrass was placed immediately into plastic tanks (415 mm long, 270 mm wide, and 255 mm high), where it remained for the duration of the isotopic enrichment experiment. The seagrass was predominantly the long-leaved *Z. capricorni* morph (200 to 300 mm long, approximately 5 mm wide) as identified by O'Donohue et al. (1991). Seagrass shoot density averaged 425 shoots m^{-2} (n = 20).

At the same time as seagrass turf was collected for the enrichment experiments, 4 samples of the following material were collected for the measurement of natural abundance stable isotope values: (1) seagrass, cleaned of epiphytes; (2) seagrass, with epiphytes attached; (3) epiphytes removed from the seagrass.

Laboratory procedures: All tanks of seagrass turf were retained indoors for the duration of the isotopic enrichment experiments. Four tanks of seagrass were contained within 1 large holding container. The volume of water in individual tanks ranged from 11 to 19 l, depending on the amount of seagrass substrate contained in each tank. Each tank was connected to a separate flow-through seawater system. Seawater was pumped from 2 km offshore in Moreton Bay, and was sand filtered before it reached the tanks. The flow rate into each tank was adjusted to a rate of about 11 l h^{-1}. Fluorescent lights attached directly above the tanks provided a light intensity of approximately 400 µEinst m^{-2} s^{-1} at the water surface from 06:00 to 18:00 h. Water temperatures were maintained between 25 and 27°C.

The ^{15}N was added as ^{15}N-enriched potassium nitrate (>98% ^{15}N). The enriched substrate was dissolved in 5 ml of distilled water, and then distributed over the surface of each individual tank.

Short-term ^{15}N enrichment: The aim of this experiment was to gain the greatest separation between seagrass and epiphyte $\delta^{15}N$ values within a 1 h period by examining the effects of 2 factors: (1) the time that each tank is exposed to the ^{15}N-enriched addition with no flowing seawater (the spiking period), before flushing with a faster than normal flow rate, and (2) the concentration of ^{15}N-enriched potassium nitrate ($K^{15}NO_3$).

Four combinations of spiking period and flushing time (amount of time the water was turned on at a faster rate to remove enriched water) and 2 substrate concentrations (low = 0.35 mg l^{-1} $K^{15}NO_3$ or 3.25 µM N; high = 0.7 mg l^{-1} $K^{15}NO_3$ or 6.5 µM N) were tested (Table 1). Three tanks were randomly assigned to each treatment, with the proviso that each large holding container had only 1 tank of each treatment.

Following the addition of ^{15}N-KNO_3 to each tank, the flow was turned off for the duration of the spiking period, and a plastic stick was used to thoroughly mix the body of water for approximately 5 s, to ensure an even mix of the enriched material throughout the water. Except for those tanks collected immediately after 15 min (Combination 1), the flow-through seawater system was turned on at a faster rate (the flushing rate of approximately 80 l h^{-1}) after the spiking period (Table 1). All other combinations were collected 1 h after the initial spike, and were then placed into plastic bags.

Longer-term ^{15}N enrichment using multiple additions: The second experiment was designed to examine 2 aspects of ^{15}N enrichment of seagrass and epiphytes: (1) to determine which ^{15}N-addition interval (every 3 or 6 d) creates the greatest and most consistent separation between the $\delta^{15}N$ values of seagrass and epiphytes over an 18 d period, and (2) to examine the loss of enrichment (or decay) of seagrass and epiphyte $\delta^{15}N$ values after 1 initial addition.

Three treatments were examined: (1) 1 initial ^{15}N addition only (i.e. decay tanks); (2) ^{15}N additions every 3 d; and (3) ^{15}N additions every 6 d. Samples of seagrass, epiphytes and seagrass with epiphytes were collected from the 3 treatments at various intervals over 18 d (Table 2). Three tanks were assigned to each collection time for each treatment. Two-way analysis of variance (ANOVA), followed by Tukey pairwise comparisons where appropriate, were used on log-transformed isotope ratios to test for significant differences between seagrass and epiphyte values (seagrass with epiphytes attached was excluded) and amongst times.

The initial concentration of ^{15}N-enriched potassium nitrate added was 0.7 mg l^{-1}. All subsequent ^{15}N additions were 0.5 mg l^{-1}. Following each addition, flowing water was turned off for 15 min, followed by a fast flushing rate for another 45 min to remove the enriched water. After this time, the flow-through seawater sys-

Table 1. Combinations of spiking period, flushing time and sampling time

Combination	Spiking period	Flushing time	Sampling time (after initial ^{15}N-addition)
1	15 min	–	15 min
2	15 min	45 min	1 h
3	30 min	30 min	1 h
4	1 h	–	1 h

Table 2. Sampling days for 3 ^{15}N-addition treatments

Treatment	Day of tank sampling
(1) 1 initial ^{15}N-addition	1
	3[a]
	6[b]
	18
(2) ^{15}N additions every 3 d	3[a]
	6
	12
	18
(3) ^{15}N additions every 6 d	6[b]
	12
	18

[a]Treatments 1 and 2 share the same tank data for Day 3
[b]Treatments 1 and 3 share the same tank data for Day 6

tem was returned to the normal slow rate of approximately 11 l h^{-1}.

Sample collection and preparation: At each sampling time, the following were collected: (1) seagrass, cleaned of epiphytes; (2) seagrass with epiphytes attached, and (3) epiphytes only. All seagrass leaves were removed at the shoot base and placed in a plastic bag. Leaves were then gently washed in distilled water. Some diatoms may have been lost during the rinsing process, but rinsing was necessary to remove salt and excess detritus (Ott 1990). Epiphytes were removed by gently scraping the seagrass leaves with a dull razor blade. This scraping method was used instead of the acid wash technique (5% hydrochloric acid) (e.g. Kitting et al. 1984), which can alter the nitrogen composition and $\delta^{15}N$ isotope ratios of seagrass (Bunn et al. 1995). The epiphyte sample was collected in a small amount of distilled water in an aluminum foil container. Epiphytes consisted of 3 main types: encrusting calcareous red algae, short filamentous algae and diatoms.

Stable isotope analysis: All samples were dried in an oven at 60°C for 24 to 48 h. Enriched and natural abundance samples were dried in separate ovens to reduce the risk of contamination. All natural abundance samples were ground in a ring grinder. Small amounts of the dried samples from the enrichment experiments were ground using a porcelain mortar and pestle. After each sample was crushed, the mortar and pestle were washed in 10% hydrochloric acid, and then rinsed twice in distilled water to prevent contamination.

Dried samples were weighed and analysed for $^{15}N/^{14}N$ and %N, using a continuous flow-isotope ratio mass spectrometer (Europa Tracermass, Crewe, UK). Ratios of $^{15}N/^{14}N$ were expressed as the relative per mil (‰) difference between the sample and recognised international standards (N_2 in air). These differences are expressed in δ-notation:

$$\delta^{15}N = \left(\frac{^{15}N/^{14}N_{sample}}{^{15}N/^{14}N_{standard}} - 1 \right) \times 1000 (‰)$$

Results. ***Short-term ^{15}N enrichment:*** Mean $\delta^{15}N$ values for seagrass and epiphytes from the first experiment (Fig. 1) were enriched (seagrass $\delta^{15}N$ range = 25 ± 4 to 90 ± 17‰; epiphyte range = 87 ± 29 to 713 ± 118‰) compared to the natural abundance $\delta^{15}N$ values from the field (3.7 ± 0.2 and 3.8 ± 0.4‰ respectively). The uptake of ^{15}N by seagrass and epiphytes was evident within 15 min of adding the enriched substrate (Combination 1). The $\delta^{15}N$ values for seagrass and epiphytes continued to increase after the 15 min spiking period despite a flushing rate of 80 l h^{-1} (Combination 2). Seagrass with epiphytes attached, and epiphytes alone were generally more enriched after the addition of the high concentration than the low concentration. The proportional differences between the $\delta^{15}N$ values for epiphytes at the high and low concentrations were greater than those observed for seagrass, except for Combination 3. In tanks with a high concentration added, epiphyte $\delta^{15}N$ values were generally double those spiked with the low concentration. The $\delta^{15}N$ values for seagrass with epiphytes attached were intermediate between those of seagrass and epiphytes.

Large differences between the $\delta^{15}N$ values of seagrass and epiphytes (>200‰) were recorded in all

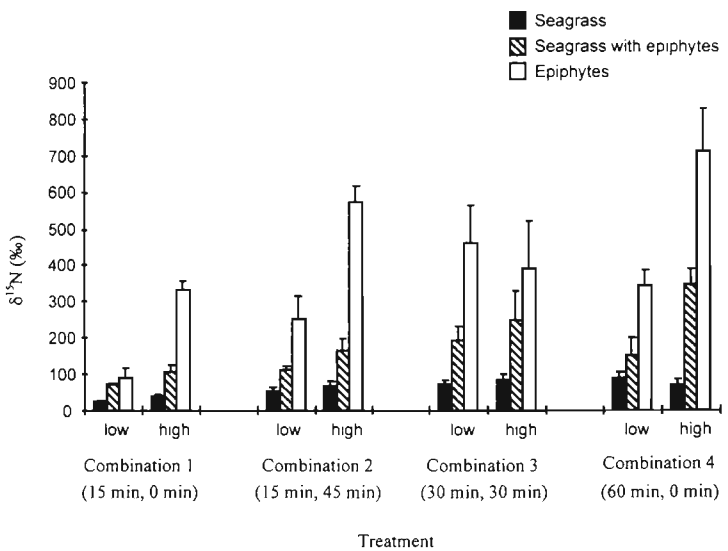

Fig. 1. Mean $\delta^{15}N$ values (+1 SE) for seagrass, seagrass with epiphytes, and epiphytes using 2 substrate concentrations (low = 0.35 mg l^{-1}, and high = 0.7 mg l^{-1} of ^{15}N-enriched potassium nitrate), and 4 spiking period and flushing combinations. Duration of spiking and flushing are shown in parentheses (see Table 1 for details); n = 3 for each mean

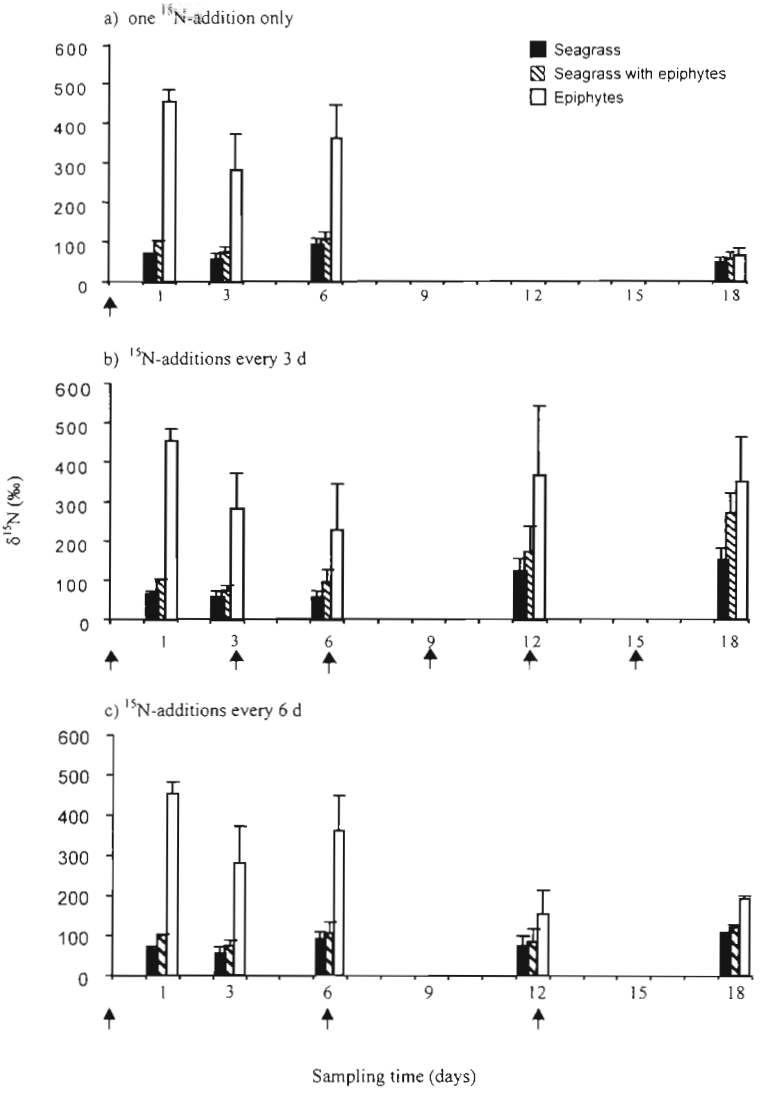

Fig. 2. Mean δ^{15}N values (+1 SE) for seagrass, seagrass with epiphytes, and epiphytes for the 3 ^{15}N-addition treatments (see Table 2 for details). ↑: ^{15}N addition. Samples collected at each time had not been respiked at that time. No samples were taken at Day 9 or Day 15

spiking periods at the high substrate concentration (Fig. 1). However, a spiking period of 15 min at a high concentration was chosen for the second experiment involving repeated ^{15}N additions because this combination resulted in good separation of seagrass and epiphyte ^{15}N signatures. It also offered a lower risk that seagrass, which we considered would retain an enriched signature for longer than epiphytes, would increase in their δ^{15}N values over time.

Long-term ^{15}N enrichment using multiple additions: As with the first experiment, the δ^{15}N values of seagrass and epiphyte samples in the second experiment (seagrass δ^{15}N range = 46 ± 13 to 149 ± 31‰; epiphyte range = 64 ± 19 to 452 ± 31‰) were more enriched than natural abundance samples in the field (4.2 ± 0.02 and 4.1 ± 0.81‰ respectively). The mean δ^{15}N values for epiphytes were higher than those for seagrass in all treatments at all times (Fig. 2).

Following a single addition of ^{15}N-enriched substrate on Day 0, the epiphyte δ^{15}N signature ranged from 279 to 452‰ until Day 6, before decreasing to 64‰ by Day 18 (Fig. 2a). The seagrass δ^{15}N signature only declined from 96‰ at Day 6 to 49‰ by Day 18. The δ^{15}N values of seagrass with epiphytes attached generally more closely resembled those of seagrass than those of epiphytes. ANOVA results demonstrated a significant interaction between plant type and day ($p = 0.033$): epiphytes had significantly higher δ^{15}N values than seagrass at all times, except Day 18.

In the treatment with multiple ^{15}N additions every 3 d, the mean δ^{15}N values for epiphytes always exceeded 200‰, and were significantly higher than seagrass values ($p < 0.001$). The δ^{15}N values of seagrass ranged from 60‰ over the first 6 d to 150‰ at Day 18, but differences among days were not significant ($p = 0.122$) and there was no significant interaction ($p = 0.373$).

The δ^{15}N values for seagrass in the 6 d ^{15}N addition treatment ranged from 64 to 101‰ over the 18 d (Fig. 2c), whereas those of epiphytes were always significantly higher ($p < 0.001$), ranging from 160 to 450‰. Differences among days were not significant ($p = 0.094$), nor was there a significant interaction ($p = 0.053$). The differences between the δ^{15}N values of seagrass and epiphytes at Days 12 and 18 were greater with a ^{15}N addition every 3 d (245 ± 145‰ and 197 ± 88‰ difference at Days 12 and 18 respectively) than with an addition every 6 d (83 ± 34 and 89 ± 13‰).

Discussion. Nitrogen has been considered a limiting nutrient in some seagrass meadows (Hillman et al. 1989), and in the present study it was taken up within 15 min of the ^{15}N addition by both seagrass and epiphytes. Within 1 h, epiphytes were considerably more ^{15}N enriched than seagrass. Algae have been found to take up nitrogen from the water column more efficiently than seagrass, possibly because algae rely totally upon nutrients from the water column, whereas seagrasses also take up nutrients from the sediment (Paling & McComb 1994). Epiphytes are also highly productive, with faster turnover times compared to

seagrass leaves (Klumpp et al. 1992, Pollard & Kogure 1993). They would therefore replace a greater proportion of their biomass with ^{15}N more quickly than seagrass. The higher surface area to volume ratio of the epiphytic algae probably also contributes to the rapid divergence in $\delta^{15}N$ values for seagrass and their epiphytes. The substantial increase in the $\delta^{15}N$ value of epiphytes after 45 min of flushing (Combination 2) indicates that ^{15}N was still available for uptake by plant sources despite the flow of new water through the tanks. This is probably due to ^{15}N availability decreasing in tanks under an exponential dilution function. However, the seawater inlets of the tanks were near the surface of the water, and it is possible that only the top few centimetres of water were constantly replaced and some of the enriched water was still available for uptake.

The high turnover of the epiphytic communities (e.g. Morgan & Kitting 1984, Pollard & Kogure 1993) probably explains the faster decrease in the epiphyte $\delta^{15}N$ compared with seagrass. After 18 d, however, epiphytes were still enriched by approximately 50‰. The regeneration of ^{15}N-nitrogen (Valiela 1984), and subsequent uptake by epiphytes, or the direct exchange of nutrients between the seagrass host and epiphytes (McRoy & Goering 1974), may have contributed to the continual enrichment of epiphytes. More recent work, however, indicates that the direct exchange of nutrients between seagrass and their epiphytes is not as important as initially thought (Brix & Lyngby 1985). The algal tissue ^{15}N content after 18 d should also represent an exponential dilution by the uptake of new ^{14}N, whereby the longer-lived algal species should still have residual ^{15}N from the initial spike.

In the second experiment, epiphytes were generally more enriched with ^{15}N additions every 3 d, compared to every 6 d, while the seagrass signature remained relatively low in both treatments. This highlights the importance of adding the enriched substrate more frequently than every 6 d, to maintain the greatest separation between seagrass and epiphyte $\delta^{15}N$ values. More frequent enrichment is also needed to maintain a relatively constant $\delta^{15}N$ value for epiphytes.

The results of the present study highlight the ability of enriched stable isotope tracers to discriminate between the $\delta^{15}N$ signatures of seagrass and epiphytes, 2 potential food sources which are often indistinguishable in their natural $\delta^{15}N$ values in the field. In trials subsequent to the experiments reported here, we placed juvenile tiger prawns (= shrimp *Penaeus esculentus*) in tanks with seagrass and epiphytes, where enriched ^{15}N was added in the same way as in these experiments. Over periods from 6 to 24 d, the prawns also became enriched in ^{15}N ($\delta^{15}N$ values increased from 10‰ to values of up to 100‰). Ultimately we aim to use this technique to determine the relative importance of seagrass and epiphytes as food sources for juvenile *P. esculentus*, a species known to feed on seagrass seeds (Wassenberg 1990, O'Brien 1994). When developed and refined, enriched stable isotope tracers have the potential to resolve food web questions that cannot be answered by natural abundance levels of stable isotopes alone.

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