Potential of ^{14}N isotope enrichment to resolve ambiguities in coastal trophic relationships

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ABSTRACT: Isotopic tracer additions were used in a field setting to differentially label primary producers within seagrass communities and trace the flow of nitrogen to consumers. Tracer experiments were coupled with fertilisation treatments, as the tracer was added to the system via a ^{15}N-depleted (i.e. ^{14}N-enriched) fertiliser. ^{15}N-depleted fertiliser was added to the sediments of *Halodule wrightii* beds to target the response of the seagrass alone, while addition to the water-column targeted the response of the seagrass/epiphyte complex. After 21 d of exposure to either fertilisation strategy, epiphytes and seagrass tissues in *H. wrightii* beds were strongly labeled with the tracer, but the mean δ^{15}N value of epiphytes (–78‰) was only significantly lower than that of seagrass (–31‰) in beds exposed to water-column fertilisation. Isotopic label was also detected in individuals of *Tozeuma carolinense*, *Farfantepenaeus aztecus*, and *Palaemonetes pugio*. The ability to both generate differential labeling in *H. wrightii* and its epiphytes and trace that label to consumers suggests that water-column fertilisation experiments incorporating ^{15}N tracers may be an excellent tool for resolving trophic relationships within aquatic communities and determining how they respond to eutrophication.

KEY WORDS: ^{15}N tracer · Water-column fertilisation · Food webs · Trophic dynamics · Seagrass communities · Epiphytic algae · Stable isotopes

INTRODUCTION

Although stable isotope approaches have been used widely in aquatic systems (Peterson et al. 1997, Mulholland et al. 2000a, Moncreiff & Sullivan 2001, Bastow et al. 2002), identifying trophic pathways from the results of stable isotope analyses can sometimes be highly problematic. Several scenarios exist in which it is impossible to determine the absolute trophic relationships between organisms from their isotopic compositions with any degree of certainty. First, if multiple food sources have the same isotopic signatures, determining the relative importance of the food sources to the consumer is not feasible. Second, when multiple sources exist (as is the common case), there is usually no unique solution indicated from the tracer information, and additional information is required to adequately assess trophic relations.

One system in which stable isotope studies have yielded ambiguous results is the trophic structure of seagrass community food webs, especially where isotopic compositions of important food resources (seagrasses and their epiphytic algae) are similar. For example, in a tropical Australian estuary, the δ^{15}N values and δ^{13}C values for *Enhalus acoroides* (Linnaeus f.) Royle and its epiphytes differed by only about 2 and 4‰, respectively (Loneragan et al. 1997). Also, δ^{13}C values for animals (–16 to –9‰) in seagrass beds of St. Croix in the U.S. Virgin Islands overlapped with values of epiphytes (–12‰) and the seagrasses *Thalassia testudinum* Banks ex König (–10‰) and *Halophila decipiens* Ostenfeld (–9‰) (Fry et al. 1982). Given these similarities in the natural abundance isotopic compositions, assessing the relative role of epiphytes versus seagrasses for consumer nutrition is difficult.

Because of these ambiguities, Winning et al. (1999) examined the effectiveness of isotopic enrichment treatments in mesocosms for generating distinct δ^{15}N values for *Zostera capricorni* Ascherson and its epiphytes. ^{15}N-enriched potassium nitrate additions were successful in separating the nitrogen isotope compositions for the 2 producers, demonstrating the ability of isotopic enrichment to differentially label seagrasses and their epiphytes in a mesocosm setting.

Although the protocol developed by Winning et al. (1999) provided a method for differentially labeling potential food sources in mesocosms, the usefulness of such a technique would be greatly enhanced if it could successfully be applied in a field setting as has been done in freshwater systems (Mulholland et al. 2000b, Evans-White et al. 2001). Differential labeling in the field could potentially elucidate the relative contribution of the various primary producer components in complex trophic networks. Additionally, field manipulations of isotopic signatures could be used to investigate other ecologically relevant questions such as determining the degree of residency and movement of mobile animals within and among seagrass beds (Fry et al. 2003) and identifying temporal or ontogenetic changes in the feeding behavior of consumers (Hentschel 1998). Given the potential of isotope labeling techniques, we conducted an experiment to test the ability of isotopic tracer additions to generate distinct isotope compositions for the seagrass, *Halodule wrightii* Ascherson, and its epiphytes in a field setting. The primary objective of the study was to develop a method for differentially labeling seagrasses and epiphytes that would permit tracking of the label to the invertebrate consumers within the system. A caveat is that the experiment was performed as a feasibility study for a larger investigation into the effects of eutrophication on the trophic dynamics of seagrass beds. This resulted in label addition occurring simultaneously with nutrient addition, rather than addition of tracer without altering nutrient levels. Results thus pertain to eutrophic rather than background conditions.

MATERIALS AND METHODS

Study site. The study site was located in seagrass beds on the northern shore of Horn Island, Mississippi, USA (30° 15.075′ N, 88° 42.602′ W). The barrier island is 11 km from the mainland, and its northern shore forms part of the southern boundary of Mississippi Sound. The tidal amplitude at Horn Island is 0.6 m. The seagrass beds in which the study was conducted were in waters of less than 2 m depth and were monospecific beds of *Halodule wrightii*. These seagrass beds are

patchy in their distribution. At the time of sampling on 6 September 2001, the salinity at the study site was 25 psu, and the water temperature was 30°C. Previous studies at the site were conducted by Moncreiff et al. (1992) and Moncreiff & Sullivan (2001).

Experimental design and sample collection. Fertilisation was targeted at the seagrass alone as well as the seagrass and epiphytes together. In order to achieve fertilisation of the seagrasses alone, fertiliser was added to the sediments in seagrass beds. Fertilisation of the water column surrounding natural seagrass beds targeted the seagrass and epiphytes together.

Nitrogen fertilisation was accomplished by adding slow-release fertiliser to each of the experimental beds. To fertilise the water column, porous tubes containing slow-release fertiliser were placed in a regular arrangement within the beds (Fig. 1). Three tubes, each containing 454 g of fertiliser (1362 g total), were placed equidistantly around the perimeter of a 1.5 m diameter circle within different natural beds. In the plots receiving sediment fertilisation, 1362 g of fertiliser pellets were gently worked into the sediments of
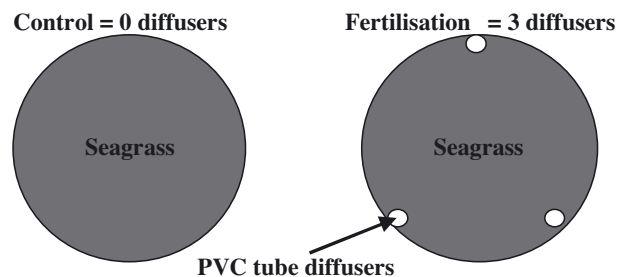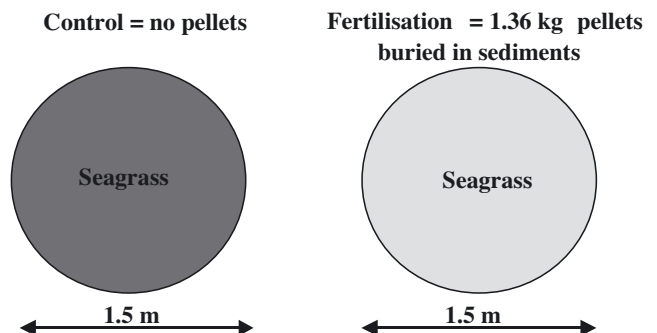

Fig. 1. Schematic representation of fertilisation strategies employed in experimental beds. (a) Water column fertilisation with diffusion tubes in seagrass beds, targeting response of both epiphytes and seagrass. (b) Sediment fertilisation, with fertiliser pellets buried in sediments of seagrass beds, targeting response of seagrass alone. All treatments were performed in triplicate

a 1.5 m diameter circle of seagrass bed in the manner described by Udy et al. (1999). In this procedure, water currents were created manually to remove the top layer of sediments from the rhizomes. The fertiliser was then spread evenly within the bed and covered with a new layer of sediment to a depth of about 1 cm. Each of these fertilisation strategies was employed in 3 beds. In addition, 3 unfertilised natural beds served as controls.

The fertiliser employed in the treatments was a custom-manufactured slow-release fertiliser (IFDC) with a chemical analysis of 19-6-12 NPK. The fertiliser was unique in that it was depleted in the heavy stable isotope of nitrogen (^{15}N) by 241‰. In order to attain this isotopic composition, the nitrogenous portion of the fertiliser was created by combining 1 part doubly-depleted ammonium-^{14}N nitrate-^{14}N with 3 parts unlabeled ammonium nitrate. A ^{15}N-depleted fertiliser was used because it was less expensive to obtain large quantities of doubly-depleted ammonium-^{14}N nitrate-^{14}N than ^{15}N-enriched ammonium-^{15}N nitrate [15]N. The fertiliser was thus relatively enriched in [14]N (not ^{15}N), so that our experiments could also be termed ^{14}N addition experiments, with low isotope δ-values expected for label uptake. The sources of phosphorus and potassium in the fertiliser were triple super phosphate and potassium chloride, respectively. All fertiliser components were mixed until homogenous and enclosed in a polyurethane coating (average of 12.7% by weight) to ensure slow release of nutrients. The coating is similar to the Polyon® coating used by Pursell Technologies on its commercial products.

Fertilisation commenced on 16 August 2001 and continued until samples were collected on 6 September 2001. Seagrass and epiphyte samples were collected by clipping a handful of seagrass blades at the sediment surface from within each of the plots. The samples were placed in bags and immediately put on ice. Upon return to the laboratory, the samples were gently rinsed with distilled water to remove any salts. The epiphytes were separated from at least 10 seagrass blades by scraping them with a rubber stopper. Such mechanical removal has been shown to be an effective means of removing the epiphytes (Burkholder & Wetzel 1990). Samples that could not be immediately processed were frozen at –20°C. The scraped seagrass blades were dried at 60°C, weighed, and ground in a Wiley mill fitted with a #40 mesh delivery tube. For collection of the epiphytes, glass-fiber filters were precombusted in the muffle furnace at ~500°C for 1 h and then weighed. Filters containing epiphytes were oven-dried at 60°C and weighed.

Invertebrate samples were collected via either dip net sweeps within the experimental beds or removal from the beds by hand. Samples were put in bags and

iced until they were transported to the laboratory. The samples were frozen at –20°C until they could be processed. After thawing, the samples were rinsed in distilled water. Wherever possible, muscle tissue was used for stable isotope analysis to obtain consistent and comparable samples for each species. If necessary, individuals collected from within an experimental bed were pooled to obtain sufficient material for analysis. Due to variability in their distribution, individuals of each species were not always collected in each of the experimental beds. For consumers with calcareous shells, the tissues were rinsed in a 10% HCl solution to remove any $CaCO_3$ that could contaminate the samples. Samples were oven-dried at 60°C and weighed. Tissues were ground with a Wiley mill equipped with a #40 mesh delivery tube. All ground tissue samples were placed in glass vials, sealed, and packed for shipping.

The stable isotope compositions for the samples were determined by mass spectroscopy. Sample values were compared to known standards, and the difference between them was recorded in per mille (‰). The formula for determining the difference is:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where X is ^{15}N and R is ^{15}N/[14]N. The standard for N is atmospheric diatomic nitrogen.

A Kolmogorov-Smirnov test of goodness of fit was used to determine whether the data exhibited departures from a normal distribution, and no significant departures were detected (p > 0.15). Two-way analysis of variance was used to make statistical comparisons of epiphyte and seagrass stable isotope values between the treatments and controls. The method of fertilisation was not explicitly included as a factor, because we were not interested in comparing the relative change in $\delta^{15}N$ values between sediment and water-column fertilisation; rather, we were interested in determining whether these fertilisation strategies were effective in differentially manipulating the isotope signatures of the seagrass and epiphytes with respect to the controls. Analyses could not be performed on isotopic signatures of the consumers because replicate samples were not always acquired. Fisher's protected least significant difference was used to determine differences in the means among the treatment combinations. All analyses were performed using SAS statistical software.

RESULTS

Stable nitrogen isotope compositions for *Halodule wrightii* differed among fertilisation treatments. The mean $\delta^{15}N$ value for *H. wrightii* in control beds was
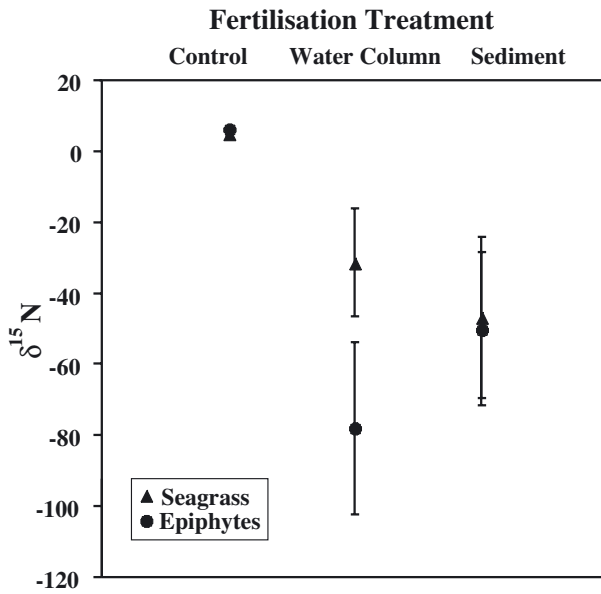
Fertilisation Treatment

Fig. 2. δ¹⁵N values (‰; mean ± 95% confidence intervals) of *Halodule wrightii* and epiphytic algae in seagrass beds exposed to control, water column, and sediment fertilisation

5.0 ± 0.7‰ (mean ± 95% confidence interval), while the corresponding values in beds receiving ¹⁵N-depleted fertiliser via the water column or sediment were –31 ± 15‰ and –47 ± 23‰, respectively (Fig. 2).

The values for both fertilisation treatments were significantly different from that of control beds (Table 1). The isotope compositions of epiphytic algae (virtually all pennate diatoms) also differed among treatments. In control beds the δ¹⁵N value for the epiphytes was 6.4 ± 0.7‰, whereas values were –78 ± 24 and –50 ± 22‰ for water column and sediment fertilisation treatments, respectively (Fig. 2). δ¹⁵N values for epiphytic algae in both fertilisation treatments were significantly different from that of control algae (Table 1).

In addition to differences between epiphytes and seagrasses receiving ¹⁵N-depleted fertiliser and their respective controls, the δ¹⁵N value for epiphytes was significantly more negative than that for seagrass (Table 1, Fig. 2) within water column fertilisation beds. Thus, epiphytes and seagrass tissues in water column fertilisation beds were differentially labeled by the isotopically depleted fertiliser. Epiphyte and seagrass isotopic compositions did not differ significantly in control beds or in beds receiving sediment fertilisation (Fig. 2).

δ¹⁵N values of consumers were variable within beds receiving ¹⁵N-depleted fertiliser. In sediment fertilisation beds, little isotopic label was incorporated into tissues of consumers. For brown shrimp *Farfantepenaeus aztecus*, 2 of 3 individuals collected from sediment fertilisation beds had positive δ¹⁵N values (8.4 and 10.8‰) (Table 2) that were indistinguishable from those of 2 *F. aztecus* individuals collected from control beds (8.5

Table 1. Results of 2-way ANOVA testing effects of fertiliser depleted in ¹⁵N on δ¹⁵N values of seagrass and epiphytes. *Significant p-values (α = 0.05). Fisher's protected LSD (α = 0.05) was used to compare treatment combination means. (CE = control epiphytes; CS = control seagrass; FE = fertilised epiphytes; FS = fertilised seagrass)

Factor	df	SS	MS	*F*	p	Conclusions
Water column fertilisation of natural beds						
Fertilisation	1	10926	10926	67	0.0001*	
Primary producer	1	1544	1544	10	0.01*	
Fertilisation × Producer	1	1726	1726	11	0.01*	(CE = CS) > FS > FE
Error	8	1274	159			
Sediment fertilisation of natural beds						
Fertilisation	1	8791	8791	46	0.0001*	(CE = CS) > (FS = FE)
Primary producer	1	3	3	0.02	0.9	
Fertilisation × Producer	1	16	16	0.08	0.8	
Error	8	1543	193			

Table 2. δ¹⁵N values of invertebrate consumers collected in individual experimental beds. Values in parentheses indicate number of individuals constituting a pooled sample; all other values are for a single individual. na: not applicable

| | Fertilisation strategy | | |
	Control	Sediment	Water column
Farfantepenaeus aztecus	+8.5, +10.3	–0.6, +8.4, +10.8	na
Palaemonetes pugio	+10.1 (16)	–9.1 (4); +7.8 (9)	–10.7 (3)
Tozeuma carolinense	+8.6 (4)	na	–1.4 (2); –3.7 (11); –12.1 (7)+1

and 10.3‰). The remaining individual collected from a sediment fertilisation bed, however, had a $\delta^{15}N$ value of −0.6‰, consistent with tracer uptake.

Pooled *Palaemonetes pugio* individuals from a sediment fertilisation bed had a $\delta^{15}N$ value similar to that of pooled individuals from control beds (7.8 and 10.1‰, respectively) (Table 2), while the $\delta^{15}N$ value of pooled individuals from a different sediment fertilisation bed was −9.1‰.

Detection of label in consumers from beds receiving water column fertilisation was more consistent. Pooled *Palaemonetes pugio* individuals from a control bed had a $\delta^{15}N$ value of 10.1‰, whereas pooled individuals from a water column fertilisation bed had a value of −10.7‰ (Table 2). In addition, the pooled isotopic signature for arrow shrimp, *Tozeuma carolinense*, was 8.6‰ in a control bed, but its $\delta^{15}N$ value in each of the 3 water column fertilisation beds was negative (−1.4, −3.7, and −12.1‰).

DISCUSSION

The strongly negative isotope compositions of both *Halodule wrightii* and its epiphytes clearly demonstrate the incorporation of our tracer by the primary producers. Tracer uptake was clearly evident in both sediment and water column fertilisation treatments, suggesting that either fertilisation method may be effective for manipulating seagrass and epiphyte isotope compositions. In our experiments, the mean $\delta^{15}N$ values of seagrass declined by 36‰ in water column fertilisation plots, and 52‰ in sediment fertilisation plots. The net changes in seagrass $\delta^{15}N$ values for both water column and sediment fertilisation plots are within the ranges reported by Winning et al. (1999) for short-term experiments (net change of 21 to 87‰). The net change for seagrass in sediment fertilisation plots also falls within the range reported by these authors for long-term experiments (net change of 42 to 145‰). For epiphytes, declines of approximately 84‰ in water column fertilisation plots and 56‰ in sediment fertilisation plots were found. The net change for epiphytes in water column fertilisation plots is at the low end of the range (83 to 710‰) reported by Winning et al. (1999). This change represents a substantial departure from natural abundance values.

In considering our $\delta^{15}N$ values, however, it is important to account for additional factors that likely influenced the observed signatures of seagrass and epiphytes. One would expect that at equilibrium, the $\delta^{15}N$ values of the seagrass and epiphytes would approach the fertiliser value of −241‰. However, based on turnover rates, it is likely that the isotopic compositions of the epiphytes reached equilibrium. Reported divi-

sion rates of benthic salt marsh and estuarine diatoms range from 0.2 to 3.2 divisions d⁻¹ (Williams 1964, Admiraal 1977). Even at the lower division rate, epiphytic diatoms would have completed 4 generations in the 21 d experiment. On the other hand, leaf turnover times for *Halodule* species range from 20 to 45 d (Hemminga et al. 1999). Because the life span of a leaf, site of most of the N uptake, equals or exceeds the duration of the experiment, it is less likely that steady state was achieved in the *H. wrightii* leaves. As a result, one would expect $\delta^{15}N$ values to decline further with continued fertilisation. Another factor that would prevent isotope signatures from approaching −241‰ is the uptake of nitrogen from sources other than our fertiliser. Undoubtedly, unlabeled nitrogen is present in both the water column and sediments of the experimental beds. While previous experiments with these diffusion tubes indicates that they do in fact elevate local water column nitrogen levels (Wear et al. 1999), it is impossible in such open systems to insure that the fertiliser is the only nitrogen source for the primary producers. That being the case, epiphytes and seagrass likely acquired unlabeled nitrogen from the water column. In addition, seagrass root uptake of sediment nitrogen would to some degree dampen the effects of our fertiliser addition on the isotopic composition. Finally, the clonal nature of a *H. wrightii* bed makes it possible for ramets in unfertilised areas to share nitrogen with ramets in fertilised areas and vice versa. Such sharing could affect label acquisition of seagrass in our fertilised beds. Regardless of the influence of these factors on the isotopic signatures, the observed decreases in $\delta^{15}N$ values we were able to produce could be very useful for investigating various ecological questions within these communities. Indeed, the entire range of $\delta^{15}N$ values found here for both seagrasses and epiphytes in fertilised treatments is absolutely distinct from $\delta^{15}N$ values of all primary producers reported over a 2 yr period for the same locality by Moncreiff & Sullivan (2001).

Results of the water column fertilisation experiments suggest that the isotope addition strategy employed may be most useful for investigating trophic relationships within seagrass communities. These experiments are relatively simple to set up, and the mean $\delta^{15}N$ value of the epiphytes was more depleted than that of the seagrass by a factor of 2. This large difference in $\delta^{15}N$ values should make it possible to identify the relative importance of these producers in the diets of consumers within the system by direct examination of $\delta^{15}N$ values of the consumers.

In the sediment fertilisation plots, the results were contrary to our expectations. Originally, it was hypothesized that fertilising sediments would only affect the isotopic composition of the seagrass, as they have

direct access to nutrients in the sediments. Based on this hypothesis, we expected that the isotopic composition of the seagrass would be depleted as a result of nutrient uptake by the roots and rhizomes, but the composition of the epiphytes would be unaltered. We found, however, no separation between $\delta^{15}N$ values of the 2 primary producers in the sediment fertilisation treatments. Two possible explanations exist for the depletion of $\delta^{15}N$ values of epiphytes. Epiphytic uptake of nitrogen that was 'leaked' by the seagrasses, as has been documented for carbon and phosphorus from *Zostera marina* Linnaeus (Penhale & Thayer 1980), could conceivably lead to a convergence in $\delta^{15}N$ values. Alternatively, and perhaps more likely, the epiphytes could be taking up nutrients that were leached directly from the sediments to the water column. Direct uptake of nitrogen from the water column would cause a decrease in the isotopic composition of epiphytes as was demonstrated in water column fertilisation treatments.

In regards to isotopic compositions of the consumers, the most interesting result is that some transfer of label up the food web was observed within the 21 d period. This result was surprising in that one might assume that such animals are too mobile to acquire any signal at all. While the presence of signal varied among fertilisation strategies, the negative isotope compositions for *Palaemonetes pugio*, *Farfantepenaeus aztecus*, and *Tozeuma carolinense* indicate that these consumers were, in fact, feeding on primary producers within our experimental beds. At this point, it is impossible to determine the extent to which they consumed seagrass tissue, epiphytes, or producers whose $\delta^{15}N$ values were not determined, such as the phytoplankton or sediment microalgae. Regardless, the results suggest that the residence time of organisms within an individual seagrass bed may be higher than expected. Differences among consumers in their residence time within a seagrass bed may explain differences in the observed degree of label uptake. The fact that all samples of *T. carolinense* exhibited label may indicate that this species has a greater residence time than the other shrimp species.

It is likely that the isotopic composition of consumers was still changing in response to changes in the composition of the food sources. As a result, the composition of the consumers may lag behind that of the seagrass and epiphytes. In a mountain stream, McCutchan & Lewis (2002) found that algal isotopic composition changed seasonally, and the composition of several consumers followed these changes but lagged behind by as much as several weeks. Based on these results, a prediction for future work within seagrass beds is that organisms feeding on the rapidly labeled epiphytic algae would acquire the label more quickly than organisms feeding on seagrass. Sampling over an adequate time course could then be useful in identifying the importance of various sources of organic matter.

Our success in labeling primary producers and demonstrating the transfer of the label to the next trophic level strongly suggests that our methodology is a viable approach for answering a suite of ecologically relevant questions within seagrass systems. Long-term addition of stable isotope labels could be used to investigate the degree of residency and movement of organisms within and between beds as well as to identify the nature and strength of trophic relationships in the system. We are currently employing this technique on a long-term (>90 d) basis to estimate the relative trophic importance of seagrass and epiphytes in the system, and to determine their response to simulated eutrophication. In these experiments we will be able to address many of the questions raised in this study, including how long-term fertilisation affects organismal $\delta^{15}N$ values.

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