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Near-Infrared Reflectance Spectroscopy is a Rapid, Cost-Effective Predictor of Seagrass Nutrients

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Abstract Near-infrared reflectance spectroscopy was used to analyze nutrient composition of tropical and subtropical seagrasses in Queensland, Australia, as part of a broader study of impacts of grazing by dugongs on seagrass. Seagrass samples of 10 species were collected, transported to the laboratory, and separated into leaf and root/rhizome fractions. They were dried, ground, and near-infrared spectra (400–2500 nm) were collected. We used partial least-squares regression to develop calibration equations relating spectral data to standard compositional analyses performed in the laboratory. These compositional analyses focused on attributes believed to be important determinants of nutritional quality of marine vertebrate herbivores (nitrogen, organic matter, neutral detergent fiber, acid detergent fiber, lignin, neutral starch, water-soluble carbohydrates, and *in vitro* dry matter digestibility). Calibration equations for each attribute were developed separately

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for (1) roots/rhizomes and (2) leaves, irrespective of plant species. An equation that combined both plant parts was equally robust. These studies demonstrated the utility of near-infrared spectroscopy in providing rapid and cost-effective analysis of marine plants, which, in turn, permits a rigorous statistical approach to be applied to studies of foraging by marine herbivores.

Keywords Near-infrared spectroscopy · Seagrass · Grazing · Nutritional quality · Marine herbivores · Nutrient analyses · Ecological methods

Abbreviations

MPLS	modified partial least squares
NIRS	near-infrared reflectance spectroscopy
RMS	root mean square
SEC	standard error of calibration
SECV	standard error of cross validation
SEL	standard error of laboratory determination (=analytical precision)
r^2	simple coefficient of determination

Introduction

Analysis of nutrient content of vegetation is a key step in many ecological studies in marine environments. However, conventional methods of such analyses are frequently time consuming and expensive. In particular, replication and longitudinal experiments can be compromised because of the difficulty of conducting nutrient analyses over time. This constraint is particularly serious when studying the nutritional plant quality for those herbivorous species that may eat a variety of food plants growing under a variety of conditions.

Near-infrared reflectance spectroscopy (NIRS) offers an unrivalled potential to address these issues (Foley et al., 1998) and has become a widely used method for analyzing agricultural and food products. Advantages offered are analytical speed, no or minimal sample preparation (Osborne et al., 1993; Shenk and Westerhaus, 1993b), low cost, high precision, and suitability for longitudinal studies. Foley et al. (1998) reviewed the basics of NIRS, which essentially depends on establishing a statistical relationship between the spectrum of near-infrared light reflected by a sample and a set of standard laboratory analyses of components of interest. Defined chemical bonds absorb light at characteristic wavelengths. Once this relationship has been established, the concentration of constituents of interest in any new samples can be analyzed solely by collecting and processing spectra from the samples.

We were interested in several nutritional attributes of different species of seagrass available to herbivorous dugongs (*Dugong dugon* Müller; Mammalia, Sirenia) in tropical Australia as part of a broader study (Aragones, 1996) of the effect of grazing on seagrass community structure. That study generated 1165 samples for which we sought information on eight nutritional components—a total of 9320 determinations. This was beyond the capacity of our or any other laboratory to complete, and so we investigated whether NIRS would allow us to keep our preferred experimental designs at a much-reduced cost.

In this paper, we show that NIRS can be successfully used to analyze nutrient concentration and *in vitro* digestibility of many seagrass species sampled from diverse

habitats and localities in tropical and subtropical Australia. In addition, we evaluate implications of our results for further applications of this technique to seagrass—herbivore interactions and seagrass ecology. We conclude that NIRS can be used reliably to analyze seagrass nutrients and could be applied to many ecological studies.

Methods and Materials

Seagrass Collection

Samples of seagrasses were collected in tropical Australia including the Great Barrier Reef region and the Gulf of Carpentaria, and subtropical south Queensland (Moreton and Hervey Bays). The collection comprised 10 species (with two species having two morphs), representing the diversity of regional seagrasses: *Halophila ovalis* (R. Brown) Hooker, *H. minor* (Zollinger) den Hartog, *H. spinulosa* (R. Brown) Ascherson, *H. decipiens* Ostenfeld, *H. tricostata* Greenway, *Halodule uninervis* (Forsskal) Ascherson (narrow- and wide-leaf morphs), *Cymodocea rotundata* Ehrenberg and Hemprich ex Ascherson, *C. serrulata* (R. Brown) Ascherson and Magnus, *Syringodium isoetifolium* (Ascherson) Dandy, and *Zostera capricorni* Ascherson (narrow- and wide-leaf morphs). We included samples from several experimental treatments (grazing, cropping, and fertilization; Aragones, 1996) as well as material from unmanipulated plots established to measure variation in nutrients because of season, depth, and locality. Most samples came from intertidal beds, but a small subset was collected with SCUBA at Pipon Island (14°7′S, 144°32′E) down to 30 m. We collected a total of 1165 samples.

Sample Preparation

Samples were washed and cleaned of adherent substrate in clean seawater and sorted into species. All were divided into leaf and root/rhizome fractions, except for *H. tricostata* and *H. decipiens*, where leaves and roots/rhizomes were combined because their leaves are so small. Fractions were placed in labeled paper bags and dried to constant mass in a forced draft oven at 60°C. Each sample was ground through a 1-mm screen in a Cyclone mill (Udy Corporation, Fort Collins, CO, USA) and stored in a polyethylene jar. One week prior to collection of NIRS (see below), we opened and equilibrated samples in a chamber maintained at 11% r.h. and 12°C. This ensured that moisture content of all samples was similar (Shenk and Westerhaus, 1993b).

Routine NIRS Scanning and Analyses

Collection of Spectral Data

Near-infrared reflectance spectroscopy is commonly used in agriculture, and standard methods have been developed (Anon, 1995). In our work, we followed these standard procedures carefully and so only briefly detail our procedures.

We acquired spectra of all samples using a near-infrared reflectance spectrophotometer (Model 6500, NIR Systems, Silver Springs, MD, USA), equipped with a

spinning-cup module, housed in a room maintained at 22 ± 1°C and 55 ± 5% r.h. We acquired the mean spectrum from two 25-mm standard cells collected to a set RMS error (using first-derivative spectral data), of 50 μ absorbance units. Spectral data were collected from 400 to 2498 nm at an interpolated data gap of 2 nm. This ensured collection of spectral data of high precision (Shenk and Westerhaus, 1993b). Reflectance (R) readings were converted to absorbance (A) values: $A = \log(1/R)$. The instrument was maintained and operated, spectral data manipulated, and calibrations developed using the NIRS3 software package (Shenk and Westerhaus, 1992).

Many of our samples were too small (~0.5–2.0 g) for use of standard cells. We made three "micro" ring cups that allowed collection of spectra from these samples. These cups had the same external dimensions as standard ring cups supplied commercially (ISI, Port Matilda, PA, USA), but the internal dimensions were reduced by 70%. We made the cups from black anodized aluminum and spectrally matched, spectroscopic-grade quartz glass (Behmn, Dayton, OH, USA) in front of each cell. We routinely used "micro" cells for samples as small as 0.3 g throughout the project, once we verified spectral data for the same samples scanned in each cell type did not differ.

NIRS Analysis

Selection of the Calibration Set for Chemical Analyses

We chose a subset of samples for detailed chemical analyses (calibration set) using the algorithms CENTER and SELECT (Shenk and Westerhaus, 1991a,c). These algorithms use principal component analysis and Mahalanobis distances (Mahalanobis, 1936) to rank spectra relative to the population average. This procedure captures a subset of the full spectral (and hence chemical) variation present in the data set.

Samples for the calibration set were selected over several months because sample collection was designed to be cumulative through time due to the limitations of the study. Processing of samples was slow because each field sample had to be sorted first into different plant species, and then each into fractions (leaves vs. roots/rhizomes). The first 200 samples represented a diversity of seagrasses of various species and morphs, collected from various sites and depths. We selected 70 of these samples for initial detailed chemical analyses. Once we had acquired spectra from all 1165 samples that were collected for the studies described by Aragones (1996), we again used the SELECT algorithm to select further 118 samples. We included 10 further samples identified as population outliers by their Mahalanobis distances, bringing the final calibration set to 198 samples. In several instances, there was insufficient sample for all chemical analyses, and so we selected the nearest neighbor, again based on Mahalanobis distances, for inclusion in the calibration set.

A Comparison between Leaf or Root/Rhizome and Whole Plant-Based Calibrations

Samples selected for the calibration set were divided into two groups: leaves and roots/rhizomes. Thus, two options were possible for calibration: a separate calibration for each of the fractions of leaves or roots/rhizomes, and a whole plant calibration, combining both fractions. Accordingly, we evaluated whether calibra-

tion equations developed for a single plant part were superior to a broad-based calibration developed with multiple plant parts. Plant part calibrations were developed separately for leaves or roots/rhizomes. A broad-based calibration was developed combining both plant fractions, as well as a few detrital matter samples. We assumed that leaves and roots/rhizomes samples selected in the broad-based calibration also would have been chosen in separate selections for each plant part because they were part of the same population. Thus, they would have still been representative of each group. Grouping by plant part rather than species was the simplest possible division, as our samples included 10 separate species. Predictions from single-product calibrations (i.e., roots/rhizomes or leaves) were compared to those of the broad-based calibration equations by comparing their standard error of cross validation (SECV), r^2, regression slope, and "bias." Bias is an estimate of the mean difference between laboratory-determined and NIRS-predicted values for calibration or monitoring sets (Smith and Flinn, 1991). Bias confidence limits were set to distinguish between no bias and a bias no greater than 1.0 × standard error of calibration (SEC) with 90% confidence when using a two-tailed type 1 error probability of 0.10 (Shenk and Westerhaus, 1993a). Shenk and Westerhaus (1993a) suggested that if bias were greater than the confidence limit, the calibration set may be insufficient and should be expanded.

Chemical Analysis (Laboratory Reference Methods)

Samples in the calibration set were analyzed for eight constituents. We assayed organic matter (OM) by burning a sample in a muffle furnace at 550°C for 4 hr. Total nitrogen (N) was assayed using a semi-micro Kjeldahl method calibrated against ammonium sulfate standards. Cell-wall constituents of seagrass have been implicated as important determinants of feeding by several authors (Lanyon and Marsh, 1995a; Preen, 1995), and we measured them as neutral detergent fiber, acid detergent fiber, and acid lignin using an ANKOM plant fiber analyzer (ANKOM, Fairport, NY, USA; Komarek, 1994) following Van Soest et al. (1991). Total water-soluble carbohydrates were extracted using 80% aqueous ethanol and water (Radojevic et al., 1994) and quantified as fructose equivalents using the anthrone reaction (Jermyn, 1975). Insoluble material remaining from this extraction was analyzed for starch enzymatically using a commercial total starch assay kit (Megazyme Total Starch Kit: Megazyme, Australia). We solubilized any resistant starch with dimethyl sulfoxide prior to enzymatic treatments. Finally, we measured *in vitro* dry matter digestibility of each sample (Choo et al., 1981) in ANKOM filter bags. The *in vitro* digestibility method attempts to simulate digestive processes in herbivorous mammals using the enzymes pepsin and cellulase and is potentially useful as a way of integrating all individual assays of each sample.

Accuracy of NIRS analyses depends entirely on accuracy of analyses of the calibration set. Therefore, to ensure the quality of data in the calibration set, we analyzed all samples in duplicate and repeated those that differed by more than 2% for N and 5% for other measures. Additionally, a randomly chosen subset ($N = 20$) of laboratory samples (called the laboratory validation set) was reanalyzed in duplicate to estimate precision of analyses. All analyses were expressed on a percent dry weight basis. A correlation analysis between calibration and laboratory validation analyses (sets) was performed for each reference method.

Table 1 Composition of seagrass used to construct calibration equations

Species	Part	Nitrogen	Organic matter	Neutral detergent fiber	Acid detergent fiber	Acid lignin	Starch	Water-soluble carbohydrate	*In vitro* digestibility
Zostera capricorni	Leaf (12)	1.91 (0.05)	63.9 (0.5)	42.3 (0.7)	28.0 (0.5)	15.4 (0.4)	1.1 (0.1)	0.1 (0.0)	90.2 (0.3)
	Root/rhizome (14)	0.66 (0.03)	56.2 (0.8)	34.3 (0.7)	26.2 (0.6)	15.1 (0.6)	7.1 (0.5)	0.1 (0.0)	82.3 (0.8)
Halodule uninervis	Leaf (30)	2.9 (0.1)	68.6 (0.2)	48.2 (0.2)	32.6 (0.2)	18.9 (0.2)	1.2 (0.1)	0.1 (0.0)	90.1 (0.1)
	Root/rhizome (35)	0.84 (0.01)	65.3 (0.5)	28.3 (0.3)	20 (0.2)	8.4 (0.2)	13.7 (0.5)	0.2 (0.0)	83.7 (0.2)
Halodule spinulosa	Leaf (9)	1.10 (0.0)	64.8 (0.6)	35.1 (0.4)	27.0 (0.3)	11.2 (0.4)	1.4 (0.2)	0.1 (0.0)	89.0 (0.4)
	Root/rhizome (12)	0.63 (0.0)	68.0 (0.6)	36.4 (0.3)	26.7 (0.3)	8.8 (0.2)	0.6 (0.1)	0.2 (0.0)	86.1 (0.8)
Halodule minor	Leaf (5)	1.95 (0.07)	50.9 (0.8)	31.9 (0.6)	20.7 (0.5)	11.5 (0.5)	0.8 (0.1)	0.1 (0.0)	90.1 (0.4)
	Root/rhizome (7)	0.56 (0.03)	44.2 (0.9)	24.3 (0.5)	19.8 (0.8)	8.8 (0.6)	1.8 (0.3)	0.3 (0.0)	91.3 (0.4)
Cymodocea serrulata	Leaf (4)	1.67 (0.19)	71.2 (2.3)	46.2 (2.4)	29.2 (0.5)	15.3 (0.8)	0.2 (0.1)	0.1 (0.0)	89.7 (2)
	Root/rhizome (4)	0.75 (0.07)	73.4 (1.8)	39.5 (2.6)	30.2 (2.1)	15.8 (1.9)	1.4 (0.3)	0.6 (0.1)	81.0 (2.8)
Cymodocea rotundata	Leaf (6)	2.60 (0.1)	67.4 (1.3)	50.7 (0.7)	32.9 (0.7)	19.0 (0.8)	0.8 (0.2)	0.1 (0.0)	87.0 (1.0)
	Root/rhizome (8)	0.91 (0.0)	60.7 (1.8)	42.2 (1.4)	32.5 (1.3)	19.9 (1.0)	2.3 (1.2)	0.2 (0.1)	73.0 (1.5)
Syringodium isoetifolium	Leaf (5)	1.36 (0.11)	61.4 (1.5)	37.0 (1.1)	26.7 (1.0)	10.2 (1.0)	1.6 (0.3)	1.9 (0.5)	96.9 (0.7)
	Root/rhizome (5)	0.78 (0.05)	75.5 (4.3)	34.3 (1.2)	26.3 (1.3)	11.1 (1.4)	3.9 (0.9)	6.4 (1.5)	84.7 (0.5)
Halophila ovalis	Leaf (17)	1.73 (0.06)	56.7 (0.5)	32.3 (0.4)	21.9 (0.3)	10.8 (0.4)	0.9 (0.08)	0.1 (0.0)	93.0 (0.4)
	Root/rhizome (20)	0.62 (0.02)	49.0 (0.6)	26.5 (0.4)	21.4 (0.3)	9.0 (0.3)	1.0 (0.2)	0.2 (0.0)	92.3 (0.3)
Halophila trichostata	Whole plant (3)	1.03 (0.1)	65.9 (1.1)	33.1 (0.6)	24.7 (0.9)	8.1 (0.5)	5.6 (0.9)	0.1 (0.0)	93.5 (0.8)
Halophila decipens	Whole plant (2)	0.75 (0.0)	51.5 (0.9)	27.5 (1.3)	20.4 (1.0)	4.9 (1.0)	2.6 (0.4)	0.1 (0.0)	92.5 (0.8)

All values expressed as % dry matter: mean (SE).

NIRS Calibrations

A calibration model for each of the constituents of interest was developed by regression of spectral absorbances and chemical analyses of each sample. We assessed a number of different regression methods including multiple linear regression and partial least-squares regression, but in all instances, modified partial least-squares (MPLS) regression (Martens and Naes, 1989; Shenk and Westerhaus, 1991b,c) was superior. MPLS uses all spectral data in contrast to alternative approaches such as multiple linear regression that uses a small subset of spectral data. We applied several transformations [calculation of first derivative (Osborne et al., 1993) and detrending (Barnes et al., 1989)] to the spectral data to remove the effect of particle size differences and to minimize autocorrelations between spectral measurements. These are recommended standard procedures (Anon, 1995).

The MPLS approach requires cross validation to prevent overfitting (i.e., using too many terms in the equation) and to select the optimum number of terms for each calibration equation (Osborne et al., 1993). Cross validation involves dividing the sample set into N groups and performing calibration on $N - 1$ groups with the remaining group being used as an independent validation set. This exercise is repeated until all samples have been cross-validated and residuals of each prediction are pooled to provide an SECV. A final regression model is then fitted to the data using the number of factors determined by the cross-validation procedure. Cross validation is an efficient procedure because all samples are used for both calibration and validation and avoids the need to set aside samples for a validation set (Osborne et al., 1993; Shenk and Westerhaus, 1993a). Another advantage of cross validation is that outliers from prediction residuals are identified readily (Shenk and Westerhaus, 1991a, 1993a; Osborne et al., 1993).

Table 2 Performance of combined plant part calibration equations for estimating the nutritional composition of seagrass

Component	N	Mean	Range	SEL	SEC	SECV	SECV/SEL	r^2	Bias	Bias limit
Nitrogen	198	1.15	0.38–3.60	0.06	0.07	0.08	1.33	0.99	−0.01	0.04
Organic matter	192	61.4	32.0–77.4	2.00	1.93	2.17	1.08	0.96	0.01	1.16
Neutral detergent fiber	198	34.2	19.2–59.8	2.54	2.14	2.79	1.10	0.94	0.00	1.28
Acid detergent fiber	198	25.0	12.9–41.2	2.02	1.89	2.15	1.06	0.91	−0.02	1.13
Acid lignin	195	13.0	2.1–30.2	3.18	3.11	3.35	1.05	0.73	0.31	1.86
Starch	175	5.6	0.2–30.6	0.90	1.08	1.25	1.40	0.98	−0.36	0.65
Water-soluble carbohydrate	184	1.7	0.01–7.95	0.55	0.58	0.60	1.09	0.90	0.02	0.35
In vitro dry matter digestibility	198	86.7	62.7–98.1	2.48	2.52	2.76	1.11	0.86	−0.19	1.45

All values expressed as % dry matter. Number of samples used in the calibration (N), standard errors of laboratory analysis (SEL), calibration set (SEC), cross validation (SECV), ratio of SECV to SEL, bias, and limits of the bias associated with NIRS equations developed using a combined population of plant fractions (leaf + root/rhizome).

Predictions from Calibration Equations

Calibration equations obtained from MPLS regressions with cross validation for each nutritional attribute were used to predict constituent values of the entire sample population using the PREDICT algorithm of the NIRS3 software (Shenk and Westerhaus, 1992). These calibrations were monitored statistically for performance. Linear regressions of predicted values ($\hat{Y}$) vs. laboratory reference values (X) were determined for each constituent. Other statistics (r^2, SEC, SECV, bias and bias confidence limits calculated as percentage of means, and slope) were computed through NIRS3 (Shenk and Westerhaus, 1992).

Results

Details of composition of the 198 samples used in the calibration set are presented in Table 1. There was no significant difference (paired t test, range of P = 0.856–0.072; N = 20) in predicted values for any of the eight constituents for material from large samples (>2 g) scanned in standard cells or small amounts (0.3–0.5 g) of the same material scanned in micro cells. The *lower P* value (0.072) was for water-soluble carbohydrates, values for which tended to vary little, and thus there is a greater tendency for the comparisons to approach significance. We also examined correlations between the values from the large and small cells. In all cases, the correlations were very close to 1, so we are satisfied that our estimates of composition of small samples of seagrass leaf and rhizome are valid.

Table 3 Performance of plant part specific calibration equations for estimating the nutritional composition of seagrass

Component	Roots and rhizomes fraction					Leaf fraction				
	N	SECV	r^2	Bias	Bias limit	N	SECV	r^2	Bias	Bias limit
Nitrogen	102	0.07	0.92	0.00	0.03	85	0.099	0.99	0.01	0.04
Organic matter	94	1.92	0.98	0.14	0.90	70	1.92	0.96	0.09	0.88
Neutral detergent fiber	102	2.52	0.89	0.19	1.25	78	2.27	0.96	0.38	1.01
Acid detergent fiber	102	1.61	0.95	−0.20	0.75	78	2.82	0.90	0.00	1.11
Lignin	100	2.76	0.79	−0.23	1.48	74	3.97	0.72	0.44	1.67
Starch	89	1.43	0.98	−0.45	0.69	64	0.13	0.26	0.23[a]	0.07
Water-soluble carbohydrate	95	0.08	0.88	0.05	0.40	70	0.05	0.92	0.04	0.23
In vitro dry matter digestibility	104	2.78	0.91	−0.07	1.34	74	2.03	0.78	−0.26	0.96

Standard errors of cross validation (SECV), simple coefficient of determination (r^2), bias of the prediction, and limits of the bias associated with NIRS analysis of nutrient composition and *in vitro* digestibility using single plant-fraction prediction equations for root and rhizome fractions of seagrass.

[a] See results for explanation of high bias estimate.

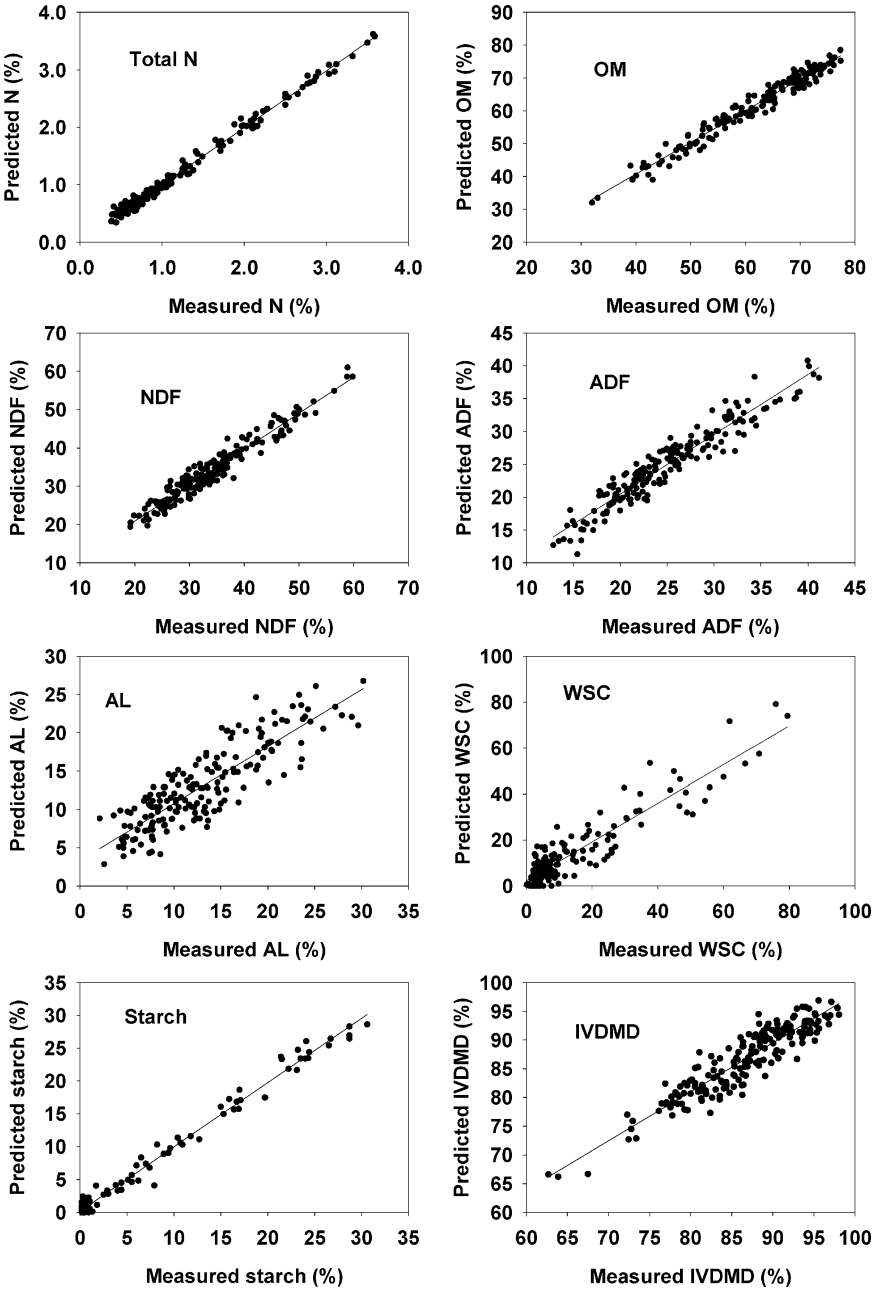

Fig. 1 Relationship between the nutritive value of seagrass measured in the laboratory and value predicted by near-infrared spectrometry. All values are expressed as % dry matter. For statistics, see Table 2

Laboratory precision [standard error of laboratory determination (SEL)] generally was excellent (Table 2) but was best for those constituents that were best characterized chemically. For example, acid lignin is a poorly defined, heterogeneous material that is difficult to analyze and had a relatively high SEL ($\bar{x} = 12.95$; SEL = 3.18). In contrast, total *N* is well defined and easy to analyze and had a relatively low SEL ($\bar{x} = 1.15$; SEL = 0.06).

Final or "global" calibration equations, developed by combining all samples irrespective of plant fraction or species, performed as well as those developed separately for roots and rhizomes (combined) and leaves (Tables 2 and 3). Only two constituents, organic matter and neutral detergent fiber, had consistently lower SECVs using calibrations based on single plant fractions than those determined from calibrations based on combining all samples. The calibration equation for starch in seagrass leaves was poor—reflected by an $r^2 = 0.26$. This was caused primarily by the small range of values for starch in leaves of seagrasses (0.2–0.8% starch of dry matter). In contrast, starch in roots and rhizomes was up to 30% of dry matter, and these data strongly influenced performance of the global equation.

The global set of MPLS-based calibration equations was superior to other regression methods in terms of SECV and coefficient of determination. Overall performance of calibration equations for the different constituents and *in vitro* digestibility of seagrasses was excellent (Table 2 and Fig. 1). This is best reflected by the closeness of the SECV/SEL ratio to 1.0. SEL values usually were low, reflecting precision of routine laboratory analyses. Proximity of values of the SECV/SEL ratio to unity (Table 2) confirms that NIRS analysis lost little precision relative to standard laboratory analytical techniques. Predicted values for all components except acid lignin agreed closely with the laboratory reference values and had r^2 values >0.90.

Discussion

This study has shown that NIRS is a suitable and powerful technique for measuring nutrient composition and *in vitro* digestibility of seagrass. The key advantage of using NIRS is that sampling regimes can be developed that satisfy statistical and biological criteria without being unduly constrained by the logistics of making traditional laboratory measurements of a large number of samples. In the context of the broader study for which these methods were developed (Aragones, 1996), only 17% of the total samples collected were subject to traditional laboratory analyses, and NIRS was used to predict the remaining 83%. This led to major savings of time and money.

Collecting duplicate spectra from a single sample takes at most 1–2 min, and, depending on how instrument software is configured, results for all calibrated constituents appear within seconds. Of course, developing calibration equations takes time, but selecting a suitable subset of samples to use in the calibration equation and validating the calibration equation take only a matter of hours in addition to standard laboratory work to chemically analyze the subset. Clearly, NIR analysis is suitable for larger data sets rather than those containing only a few samples.

A second advantage is that calibration equations are available for future studies, so that there will be no need to analyze an additional large sample set. We believe

that analysis of some samples from a future study is important to check that all new samples fall within the bounds of the existing calibration population. If not, then the calibration set can be expanded as we demonstrated here.

This study introduced several innovations that make NIRS particularly useful for ecological research in marine environments. First, development of a microsample cell demonstrated that multiple analyses could be made on small amounts of material. Sample cells for NIRS have been developed for a wide range of materials, and there is little constraint on the type and amount of material that is needed to collect spectra. In marine studies, there is often only a small amount of material available, especially from manipulative studies, and demonstration that NIRS can be used successfully for samples as low as 0.3 g of dry matter is significant. A current study (Bité and Lawler, unpublished data) is using even smaller samples with success using spinning cup inserts made by the manufacturers of the spectrophotometer.

Second, we have shown that broad-based or global calibration equations are just as good as those restricted to plant fractions. Traditionally, NIRS-based calibration equations have been narrow. For example, in analysis of protein in wheat grain, equations often are developed for wheat harvested in a single season at a single site (Batten, 1998). In contrast, especially ecological studies in marine environments usually focus on compositional changes across years, across sites, and among species (e.g., Lanyon and Marsh, 1995b). Our development of broad-based equations that incorporated leaves, roots, and detritus suggests that the method is particularly powerful for ecological studies.

Some studies (e.g., Meuret et al., 1993; Shenk and Westerhaus, 1993b) also have shown that whole plant-based calibrations differ only slightly from single-product calibrations. Smith and Flinn (1991) suggested that although development of broad-based calibrations is tedious, broad-based equations are more cost-effective than conventional techniques. This suggests that an initial broad-based calibration is more useful to develop, and expands with additional small extension populations, as required, than to gather another large set of samples for development of a specific new calibration each time this need arises. Shenk and Westerhaus (1993a) noted that development of global calibrations was made possible only by technological advances in desktop computing and software. This approach will be important for wildlife nutritional studies. The ability to limit calibrations to one equation per constituent, rather than one per constituent per plant part, will also increase the savings of adopting NIRS.

In NIRS, there always will be a trade-off between developing a robust equation of wide applicability and one that is tailored precisely to a particular set of conditions. For example, we were careful to ensure that all our samples were equilibrated to an equivalent dry matter content before scanning. This is because water has a strong absorbance in the near-infrared spectra, and this absorption can mask other absorbances that may be of interest. Similarly, we were careful to use a single, specified grinder and to keep that grinder in good order so that we produced a uniform particle size. Additional robustness of a calibration equation can be achieved by including variation for particle size and sample moisture. For example, if samples were to be prepared and scanned in several different laboratories, these issues might be accorded more weight. A small amount of precision may be sacrificed for a more robust equation, depending on the uses to which the equation is to be put. Potential users of this technique should not necessarily try to emulate the detail of the approaches that we used for equation development, but any laboratory

analyst has to think carefully about an equation's end use before deciding what analytical precision and accuracy are acceptable.

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