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THE USE OF NEAR INFRARED REFLECTANCE SPECTROMETRY FOR CHARACTERIZATION OF BROWN ALGAL TISSUE[1]

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Measuring qualitative traits of plant tissue is important to understand how plants respond to environmental change and biotic interactions. Near infrared reflectance spectrometry (NIRS) is a cost-, time-, and sample-effective method of measuring chemical components in organic samples commonly used in the agricultural and pharmaceutical industries. To assess the applicability of NIRS to measure the ecologically important tissue traits of carbon, nitrogen, and phlorotannins (secondary metabolites) in brown algae, we developed NIRS calibration models for these constituents in dried *Sargassum flavicans* (F. K. Mertens) C. Agardh tissue. We then tested if the developed NIRS models could detect changes in the tissue composition of *S. flavicans* induced by experimental manipulation of temperature and nutrient availability. To develop the NIRS models, we used partial least squares regression to determine the statistical relationship between trait values determined in laboratory assays and the NIRS spectral data of *S. flavicans* calibration samples. Models with high predictive power were developed for all three constituents that successfully detected changes in carbon, nitrogen, and phlorotannin content in the experimentally manipulated *S. flavicans* tissue. Phlorotannin content in *S. flavicans* was inversely related to nitrogen availability, and nitrogen, temperature, and tissue age interacted to significantly affect phlorotannin content, demonstrating the importance of studies that investigate these three variables simultaneously. Given the speed of analysis, accuracy, small tissue requirements, and ability to measure multiple traits simultaneously without consuming the sample tissue, NIRS is a valuable alternative to traditional methods for determining algal tissue traits, especially in studies where tissue is limited.

Key index words: **algae; carbon; near infrared reflectance spectrometry; NIRS; nitrogen; nutrients; phlorotannin; *Sargassum;* temperature**

Abbreviations: **NIRS, near infrared reflectance spectrometry; PGE%, phloroglucinol equivalents of dry weight; PLS, partial least squares regression; RGR, relative growth rate**

Plants exhibit ecophysiological and functional diversity, which underlies variation in growth rates, productivity, population and community dynamics, and ecosystem function (Ackerly et al. 2000). Within a species, plants can also exhibit phenotypic plasticity of traits in response to environmental conditions (e.g., nutrient availability, light, and temperature). Changes in environmental conditions can induce changes in the physiological processes and composition of plant tissue, which in turn can have effects on the wider ecosystem via changes to the nutritional value of those tissues as food for herbivores. Changes in the nutritional value of plant tissue can impact herbivore feeding behavior and fitness and can modify the outcomes of plant–herbivore interactions (Cruz-Rivera and Hay 2000, Hemmi and Jormalainen 2002). Therefore, measuring traits associated with plant tissue composition is important to understand how environmental change affects plant ecosystem dynamics and plant–herbivore interactions.

Over the last three decades, NIRS has been widely used to analyze the nutritional value of pastures and food products, offering the advantages of analytical speed, minimal sample preparation, low running costs, and high precision over traditional methods (Batten 1998). NIRS works on the basis that when near infrared light is flashed on a sample, it is absorbed at frequencies corresponding to characteristic vibrations of the chemical bonds within particular functional groups (Batten 1998, Foley

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et al. 1998). Frequencies not absorbed are either transmitted or reflected resulting in a reflected spectrum that contains information on the chemical composition of the sample. Quantification of tissue components with NIRS depends on the development of a statistical relationship between the spectrum of NIR light reflected by samples and a set of standard laboratory values for the components of interest. Once this relationship has been established, NIRS can be used to predict the concentration of the constituent of interest in any new sample by solely collecting and processing spectra from the new samples (Foley et al. 1998).

More recently, ecological studies have adopted NIRS to determine the chemical composition of plant tissues with the aim of predicting which plant traits affect palatability to herbivores. McIlwee et al. (2001) demonstrated that NIRS can accurately assess the chemical composition of *Eucalyptus* leaves (including nitrogen, neutral detergents, fiber, condensed tannins and total phenolics) and were able to use the results from NIRS to predict the intake of foliage from individual trees by greater gliders and possums. Woolnough and Foley (2002) validated the use of NIRS to determine the nutritive value of pasture available to the engaged northern hairy-nosed wombat over different seasons. NIRS has also been used to determine the effects of herbivores on plant tissue. Stolter et al. (2006) used NIRS to measure the nitrogen, fiber, and specific and total phenolics in subarctic willows and demonstrated that NIRS could predict the effects of moose grazing on willow leaf chemistry the following season. In the marine environment, NIRS has been used to measure important determinants of nutritional quality (including nitrogen, starch, carbohydrates, detergent fiber, and lignin) in seagrass species consumed by dugongs (Lawler et al. 2006) and to determine the effects of turtle and dugong grazing on the nutritional value of seagrass following grazing experiments (Aragones et al. 2006). However, despite the apparent diverse applications of NIRS, the use of NIRS to measure marine macroalgal traits has been limited to the measurement of alginate in the brown alga *Laminaria hyperborean* (Horn et al. 1999).

This study aimed to test if NIRS could be used to accurately and effectively measure three important traits associated with tissue quality in macroalgae: total nitrogen, total carbon, and phlorotannin content. The growth of marine macroalgae and their herbivores are frequently limited by nitrogen (Elser et al. 2007). As a consequence, the relative concentration of nitrogen to carbon in macroalgal tissue is commonly used as a proxy for nutritional value to herbivores (Yates and Peckol 1993, Cruz-Rivera and Hay 2000, Hemmi and Jormalainen 2002). Tissue carbon and nitrogen concentrations in macroalgae vary as a function of resource availability, including nutrients and light, and can vary among tissues at small scales (e.g., centimeters) (Arnold et al. 1995, Edwards et al. 2006). To understand the mechanisms governing algal growth and algal-herbivore interactions, it is important to measure these plastic plant-quality components in macroalgae.

Phlorotannins are polyphenolics found exclusively in the Phaeophyceae (brown algae) and are a subgroup of tannins that are acetate-malonate (polyketide) derived polymers of phoroglucinol (1,3,5-trihydroxybenzene) (Ragan and Glombitza 1986). These water-soluble phlorotannins have been found to occur in concentrations of up to 25% of dry weight, and along with their putative roles in cell-wall construction, phlorotannins can fulfill a number of ecological roles, such as protection from ultraviolet radiation, fouling organisms, or herbivores (reviewed in Paul et al. 2006, Amsler 2008, Paul and Ritson-Williams 2008). Phlorotannin concentrations are highly variable, varying over geographic regions (Steinberg 1986, Targett and Arnold 1998), species (Stiger et al. 2004, Fairhead et al. 2005), and individuals (Tuomi et al. 1989). Within a species, concentrations have been shown to vary with changes in salinity (Pedersen 1984), light availability (Pavia and Toth 2000), nutrient availability (Yates and Peckol 1993), season, age (Pavia et al. 2003), and tissue type (Van Alstyne et al. 1999).

In algal studies, the analysis of tissue composition can be restricted by a limited availability of algal tissue—for example, where whole individuals of algae are smaller than the amount of tissue required for analyses, or where within-alga patterns of variation result in the scale of the traits measured being smaller than the scale of the tissue required for analyses. At present, combustion analysis is predominately used for determination of carbon and nitrogen content in algal tissue, and the colorimetric Folin–Dennis method or Folin–Ciocalteus method is used to quantify phlorotannins. Although effective, these methods require relatively large amounts of tissue ($\sim$1 g dry weight) and consume the sample tissue during analysis, making further analyses of other constituents in the tissue impossible. Despite recent improvements in these methods (e.g., the development of 96-well microplate format by Zhang et al. 2006), the development of faster and more sample-efficient protocols would enhance the ability of researchers to carry out more elaborate and probing experimental designs addressing the roles of phlorotannins and nutritional traits in algae, especially in algal-mesoherbivore studies.

This study aimed to (a) develop NIRS calibration models for carbon, nitrogen, and phlorotannins in the brown alga *S. flavicans* (Phaeophyta: Fucales); and (b) determine if NIRS could detect changes in the tissue composition of *S. flavicans* created by experimental manipulation of temperature and nutrient availability. Elevated nitrogen availability has previously been shown to decrease phlorotannin

content in algae (Yates and Peckol 1993, Hemmi et al. 2005, Svensson et al. 2007), due to increasing growth rates of individuals, thereby reducing the pool of carbon-based photosynthates allocated to secondary metabolite production. We chose to manipulate temperature on the basis that, under unlimited resource conditions, production of enzymatic pathways associated with growth will increase with elevated temperatures, thereby increasing algal growth rates and reducing the carbon allocation to secondary metabolites. The experiment tested the two hypotheses that concentrations of phlorotannins will be lower under elevated nitrogen conditions and elevated temperatures.

MATERIALS AND METHODS

Study organism and collection details. *Sargassum flavicans* was collected from the rocky shore at Redcliffe, Moreton Bay, South East Queensland, Australia (27°3.2′ S; 153°06.7′ E). *Sargassum* tissue was collected from the shallow subtidal zone (1–1.5 m depth) where *Sargassum* is a dominant taxon within the algal community. To develop an NIRS calibration equation for phlorotannin content in *Sargassum*, 85 samples were collected from different *Sargassum* individuals in the field for phlorotannin content analysis. To capture the natural variation of phlorotannin concentrations in our NIRS calibration model, we collected samples every 2 weeks for 4 months (November 2007–March 2008). Samples of different tissue types were also integrated into the collection regime, including samples of exclusively young tissue, old tissue, reproductive tissue, blades, and stipes from different individuals.

To create NIRS calibration equations for nitrogen (N) and carbon (C), 75 samples were collected for total nitrogen and total carbon analysis from different *Sargassum* individuals. To capture a wide a range of total nitrogen and carbon variation found in *Sargassum*, tissue was collected over a 6-month period (November 2007–April 2008). Samples collected from the field were augmented with samples from laboratory and field experiments where nutrient availability was enriched.

Sample preparation. All samples from each calibration set (phlorotannin, N, and C) were freeze-dried in the condition they were collected and, after 48 h, removed from the freeze dryer and stored in sealed containers at room temperature. Samples were ground to a fine powder using a ball grinder and returned to sealed containers until further analysis.

In addition to the phlorotannin calibration samples from the field, a set of ''spiked'' samples was created to extend the range of the calibration equation to encompass higher phlorotannin concentrations found in winter months. This sample set was created from one large sample, which had been ground to a homogenous powder. The sample was split into 11 subsamples, and phloroglucinol (Sigma-Aldrich Pty. Ltd., Sydney, Australia), the base unit of phlorotannin, was added to each of these subsamples to create a range of different dry weight percentages of phloroglucinol. The percentages of phloroglucinol per dry weight of tissue of these subsamples were 1, 2, 3, 4, 6, 7, 8, 10, 12, 14, and 16%.These spiked samples were vigorously shaken to ensure that the added phloroglucinol was evenly mixed within each sample. The spiked samples were stored in sealed containers and added to the main phlorotannin calibration set ($n = 96$) for NIRS scanning and traditional phlorotannin analysis.

NIRS scanning. To obtain spectra for each calibration sample, samples were scanned using a near infrared reflectance spectrophotometer (Model 6500; NIR Systems, Silver Springs, MD, USA) (Horn et al. 1999, Andre and Lawler 2003). Spectral data were generated by flashing each sample with monochromatic light at 2 nm intervals across a range from 1,100 to 2,500 nm. Reflectance across this range was measured and stored using VISION software (Version 1.0; FOSS NIRSystems, Laurel, MD, USA). The software converted reflectance (R) readings to absorbance (A) values using the equation,

$$A = \log(1/R) \quad (1)$$

Absorbance values were used for all analyses and calibration development.

Chemical analyses. To obtain reference values for the phlorotannin calibration samples (including the spiked samples), samples were analyzed colorimetrically using the Folin–Ciocalteus method, with phloroglucinol as the standard, using a 96-well microplate method (Zhang et al. 2006). Standard solutions were prepared by dissolving phloroglucinol in distilled water to make a stock solution of 500 $\mu g \cdot mL^{-1}$. Serial dilutions of the stock solution were carried out to obtain standard solutions at the concentrations of 500, 200, 100, 50, 25, 12.5, 6, and 3 $\mu g \cdot mL^{-1}$. Phlorotannins were extracted by placing a known mass of each calibration sample (0.5–1.0 g) in a test tube containing MeOH-water (1:1). The pH was adjusted to two, and the sample was shaken at room temperature for 1 h (150 rpm). Tubes were centrifuged at 4,000*g* for 10 min, and the supernatant recovered. Acetone-water (7:3) was added to the residue, and extraction conditions repeated. Following centrifugation, the two extracted solutions were pooled and mixed. A 1:10 dilution of this solution was then used for the colorometric analysis. Each sample solution along with the standard solutions and controls were loaded on 96-well plates. Folin–Ciocalteus reagent and 7.5% sodium carbonate solution were added, followed by an incubation period. Absorbance was read at λ 750 nm with a plate reader (SpectraMax M2; Molecular Devices Ltd., Victoria, Australia). Based on the standard curve of the serial standard solutions spectrometer values ($R^2 = 0.97$, SE = 0.24), the phloroglucinol equivalents ($\mu g \cdot mL^{-1}$) were estimated for each sample and converted to total percent phloroglucinol equivalents of dry weight (PGE%). These PGE% values were used as estimates of the phlorotannin content of the tissue.

Nitrogen and carbon contents (% dry weight) of the calibration samples were determined by combustion. The 75 ground *Sargassum* samples were analyzed using a CHN Analyzer (model 2400; Perkin Elmer, Norwalk, CT, USA) at the Smithsonian Environmental Research Center, Edgewater, Maryland, USA.

Development of NIRS calibration models. Calibration equations for each constituent (phlorotannin, as PGE%, N, and C) were developed using regression analysis between values from laboratory analyses and NIRS spectra. The laboratory values of the three constituents from each calibration set were imported into VISION and matched with the corresponding spectra for each sample. Partial least squares regression (PLS), as recommended by Shenk and Westerhaus (1993), was used to develop an equation between the spectral absorbance and the laboratory values of samples from each calibration set within VISION. For the phlorotannin (PGE%) calibration, we tested if the spiked samples strongly influenced the slope of the calibration equation and found no significant differences ($P > 0.05$) between the regression slope with and without the spiked samples, although the strength of the regression was diminished without the spiked samples (from $R^2 = 0.96$ to $R^2 = 0.85$). The spiked samples were therefore included to increase the range of the calibration. Before PLS was performed, a subset of samples was set aside from each of the calibration sets and saved separately as a validation set using the standard procedures within VISION. Additionally, cross-validation was used to estimate the optimal number of terms in the

calibration models and to prevent overfitting as outlined by Osborne et al. (1993). Mathematical treatments that transform spectral data were carried out (Table 1), and the second-order derivative was used for all three calibration equations.

The calibration equations were selected on the basis of the coefficient of determination (R^2) and bias (difference between the mean actual value and the mean predicted value) along with estimates of the standard error of calibrations, the standard error of prediction, and the standard error of cross-validation. To test the validity of these equations, the equations were used to predict the constituent content of samples in the corresponding validation sets. The correlation values between the predicted constituent values and the known laboratory values of the validation samples were used to judge the strength of the final equations.

Effects of temperature and nitrogen availability on tissue qualities. To test the utility of the developed NIRS calibration models, field-collected *Sargassum* was grown under conditions of manipulated temperature and nitrogen availability, with the aim of generating variation in tissue composition. Nutrients and temperature were manipulated in a factorial design with two temperatures (21°C and 28°C) and four nutrient conditions (nitrogen availability). Ammonium (NH_4^+) was used as the N source as this is the most common N pollutant in many shallow marine systems (Dafner et al. 2007). The temperature treatments represented summer and winter temperatures at the field site and were in excess of those experienced by *Sargassum* in the field at the time of collection (~23°C).

Thirty-two *S. flavicans* individuals were collected from the study site at Redcliffe. After collection, plants were transported in natural seawater at ambient temperature to algal culture facilities at the University of Queensland. The algae were gently cleaned with seawater to remove visible epiphytes and adhering sediments. On the same day as algae were collected, a 2 g (wet weight) sample of the primary apical meristem was removed from each of the 32 individuals and used in the experiment. The algae were grown in 1 L Erlenmeyer flasks filled with filtered natural seawater (35‰) arranged in cooling basins ($90 \times 60 \times 45$ cm). The 2 g samples from each individual were randomly assigned to a flask, with each flask belonging to one of the eight combinations of temperature and nutrient treatments. There were four replicate algal samples per treatment combination.

The NH_4^+ concentrations were 7.1, 14.2, 28.5 μM, and a control with no added ammonium (<0.5 μM). Temperatures were adjusted to either 21 ± 2°C or 28 ± 2°C by adjusting the temperature within the cooling basins in which the experimental flasks were placed. Nitrogen was added to the cultures as NH_4Cl from a 1 mM stock solution. Experimental flasks were maintained under a photon density of 150 ± 50 μmol · $m^{-2} \cdot s^{-1}$ using a combination of halogen and fluorescent lights for a 12:12 light:dark (L:D) photoperiod. To prevent phosphorus and carbon limitation within the cultures, phosphorus and carbon were added as NaH_2PO_4 and $NaHCO_3$ to maintain concentrations of 10 μM and 3 μM, respectively. The flasks were aerated to ensure water movement and the maintenance of aerobic conditions. The pH of all flasks was monitored daily and maintained between 8.1 and 8.3. To maintain treatment conditions, water was exchanged every second day over the 8 d experiment.

After 8 d, algal samples were spun dry in a salad spinner (80 revolutions) to remove excess water before being weighed. The changes in biomass (wet weight) of algal tissue during the experimental period were measured to estimate growth. Mean relative growth rates (RGR), expressed as mg · g^{-1} · d^{-1}, were calculated according to the following equation, assuming exponential growth:

$$\text{RGR (mg} \cdot \text{g}^{-1} \cdot \text{d}^{-1}) = \text{In}(W_t - W_0)/t - 1 \qquad (2)$$

where W_0 and W_t are the initial and final wet weight biomass of the algal samples, and t is time in days.

The samples were then divided into new and older tissue. New tissue was defined as the tissue developed during experimental culture, and older tissue was the initial tissue added to the culture. After being separated, tissue samples were oven-dried for 48 h at 60°C before being ground to a fine powder using a mortar and pestle. These samples were analyzed for phlorotannin, N, and C tissue content using NIRS. All experimental samples were scanned using an NIR spectrophotometer following the same protocol used for the calibration samples. The concentration of phlorotannin, nitrogen, and carbon in the experimental samples was then estimated by the newly developed NIRS calibration equations (described above) using the PREDICT algorithm within the VISION software package.

Statistical analyses. All data were analyzed with the statistical package STATISTICA 8 (StatSoft Inc., Tulsa, OK, USA). Cochran's test was used to test data for homogeneity of variances, and data were transformed where necessary [log (phlorotannin) and $1/(\text{carbon})^2$] to meet the assumptions of normality for analyses of variance (ANOVA). Two-way ANOVA was used to determine the effects of ammonium and temperature on growth. To account for the nonindependence of the measurements of new apical and older basal tissue from each thallus, repeated measures ANOVA was used to determine the effects of temperature and ammonium availability on N, C, C:N, and phlorotannin content of *Sargassum* tissue. Age of tissue was treated as the within effect, and temperature and ammonium as the between effects.

RESULTS

NIRS calibration models. PLS regression between laboratory values and NIRS spectra produced

TABLE 1. Calibration statistics for each constituent, including the number of samples (N), range, standard deviation (SD), standard error of calibration (SEC) and cross-validation (SECV), coefficient of determination of the regression equation between laboratory values and near infrared reflectance spectroscopy (NIRS)–predicted vales (R^2), the number of principal components in the model (Terms), mathematical treatment of the spectral data (Maths: the first number is the order of the derivative function, the second is the segment length in data points over which the derivative was taken, and the third is the segment length over which the function was smoothed), and finally, the spectral range used in the regression equation.

Constituent	N	Mean	Range (% dry weight)	SD	SEC	SECV	R^2	Terms	Maths	Spectral range (nm)
Phlorotannin	74	3.23	0.4–12.9	2.80	0.60	0.65	0.96	5	2,10,10	1,100–2,200
Nitrogen	55	1.00	0.6–1.5	0.24	0.04	0.07	0.97	5	2,10,10	1,100–2,200
Carbon	52	26.48	25.0–28.4	0.95	0.39	0.50	0.84	4	2,10,10	1,100–2,400

calibration equations for phlorotannin, nitrogen, and carbon content in *Sargassum* tissue with high coefficient R^2 values and low standard errors of calibration and cross-validation (Table 1 and Fig. 1). All three calibrations showed high accuracy when used to predict the values of the known validation set (Table 2 and Fig. 2). Plots of laboratory versus NIRS-predicted content values (Fig. 2) for each constituent show tight linear relationships with high correlation values (Table 2).

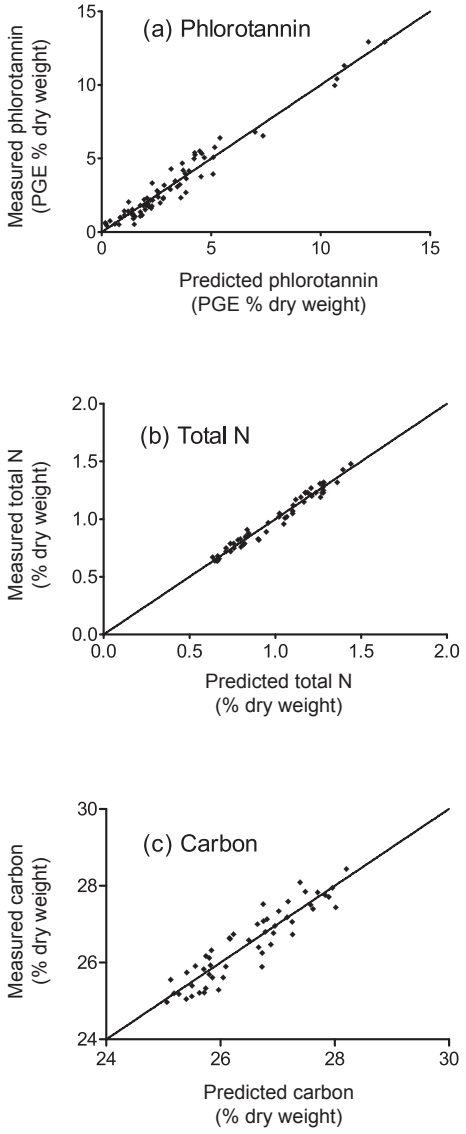

FIG. 1. Relationship between laboratory values of (a) phlorotannin, (b) nitrogen, and (c) carbon and values predicted by near infrared spectroscopy of the calibration sets. PGE% = phloroglucinol equivalents of dry weight.

Effects of temperature and nitrogen availability on tissue qualities. The development of new tissue was observed under all experimental conditions during the 8 d experiment. The addition of NH_4^+ had a significant positive effect on growth ($F_{3,24} = 7.78$, $P < 0.001$; Fig. 3) at both 21°C and 28°C. The addition of nitrogen had a significant effect on the total phlorotannin content in *S. flavicans* (Table 3; Fig. 4, a and b). Tissue grown under the highest concentration of NH_4^+ (28.5 μM) had significantly lower phlorotannin content than tissue grown under lower NH_4^+ (<0.5 and 7.1 μM) concentrations. There was a significant three-way interaction between NH_4^+, temperature, and age (Table 3; Fig. 4, a and b). New tissue grown at 21°C under ambient NH_4^+ (<0.5 μM) conditions had significantly lower phlorotannin concentrations than new tissue grown under ambient NH_4^+ at 28°C (Fisher's LSD post hoc test; Fig. 4, a and b).

Sargassum tissue grown under the highest concentration of NH_4^+ (28.5 μM) had significantly higher total nitrogen content than tissue grown under the lower NH_4^+ concentrations of <0.5 and 7.1 μM (Table 3; Fig. 4, c and d). Older tissue had significantly higher total N content than new tissue (Table 3; Fig. 4, c and d), and tissue grown at 21°C had higher total N than tissue grown at 28°C (Table 3; Fig. 4, c and d). The carbon content of *Sargassum* tissue deceased when grown under increased NH_4^+ concentrations (Table 3; Fig. 4, e and f), and new tissue had significantly higher carbon content than old tissue (Table 3; Fig. 4, e and f). The C:N ratio of *Sargassum* tissue grown at 28°C was significantly higher than tissue grown at 21°C (Table 3; Fig. 4, g and h). New tissue had significantly higher C:N ratio than old tissue (Table 3; Fig. 4, g and h), and the C:N ratio of tissue decreased with increased NH_4^+ concentrations (Table 3; Fig. 4, g and h). The C:N ratio of tissue grown under the highest NH_4^+ concentration (28.5 μM) was significantly lower than in all other treatments, and tissue grown under the intermediate NH_4^+ concentration of 14.2 μM was significantly

TABLE 2. Validation statistics, including the number of samples (*N*), the mean and range of validation set, the difference between the mean actual value and the mean predicted value (Bias), standard error of prediction (SEP), slope, standard error of slope (SE slope), and the coefficient of determination of the regression between predicted values and the validation values (R^2).

Constituent	*N*	Mean	Range (% dry weight)	Bias	SEP	Slope	SE slope	R^2
Phlorotannin	20	2.33	0.8–5.6	−0.070	0.49	0.91	0.26	0.91
Nitrogen	20	1.00	0.6–1.5	0.005	0.05	0.97	0.04	0.98
Carbon	13	26.64	25.1–28.0	−0.095	0.31	0.94	2.60	0.95

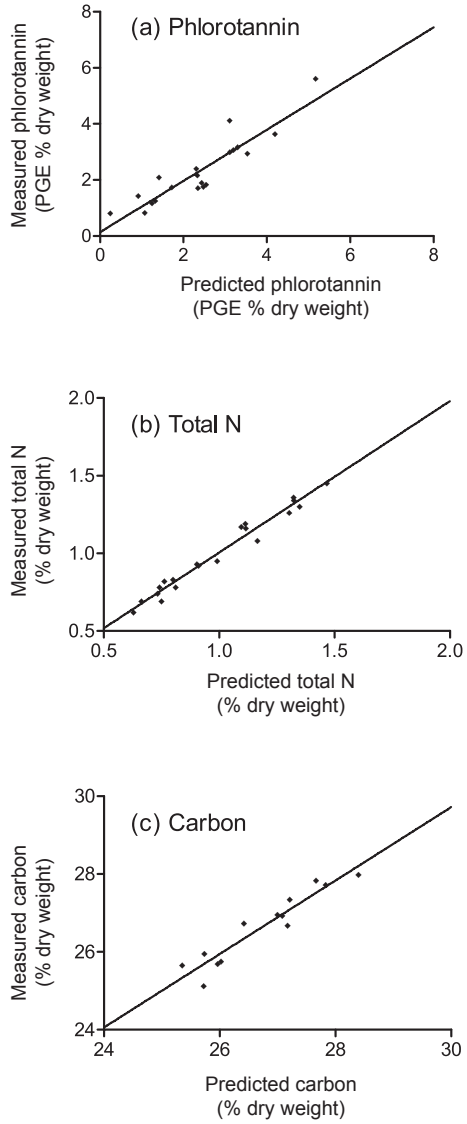

FIG. 2. Relationship between laboratory values of (a) phlorotannin, (b) nitrogen, and (c) carbon of the validation sets and the values predicted by the newly developed near infrared spectroscopy calibration models. PGE% = phloroglucinol equivalents of dry weight.

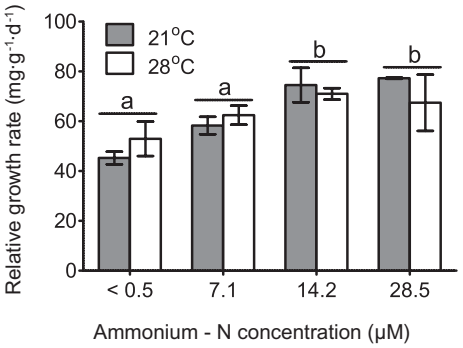

FIG. 3. Mean ± SE relative growth rate ($mg \cdot g^{-1} \cdot d^{-1}$) of *Sargassum flavicans* during the temperature and NH_4^+-enrichment experiment. Dark bars represent tissue grown at 21°C, and open bars represent tissue grown at 28°C. Different letters above bars denote significant differences between means.

lower than tissue grown under ambient (<0.5 μM) NH_4^+ concentrations (Fig. 4, g and h).

DISCUSSION

We have shown that NIRS can be used to accurately predict traits of algal tissue (nitrogen, carbon, and phlorotannin as phloroglucinol equivalents) that are fundamental for studies of physiology, ecology, and algal-herbivore interactions. We demonstrate the utility of NIRS as a time-efficient alternative to conventional methods of algal tissue analysis, which facilitates the evaluation of microscale variation in algal traits, due to the reduced amount of tissue required for analysis. We illustrate that NIRS can simultaneously measure several algal traits without consuming the sample tissue, offering advantages for future algal-herbivore experiments. We further demonstrate that this approach has the sensitivity to detect changes in algal tissue that result from variation in resource availability (temperature, nutrients), illustrating the potential of NIRS in studies investigating the effects of eutrophication and climate change on coastal algal communities.

Use of NIRS to measure algal tissue traits. The nitrogen and carbon NIRS models developed in this study match the accuracy of nitrogen and carbon models developed for other organisms in terrestrial, aquatic, and marine systems. Nitrogen models appear to be consistently accurate across different systems and tissue types. Lawler et al. (2006) developed an effective nitrogen NIRS model to quantify seagrass nutrients (R^2 of 0.99), and Hood et al. (2006) developed a useful model to measure the nitrogen content of aquatic seston samples ($R^2 = 0.87$). Calibration NIRS models for nitrogen content in pine needles ($R^2 = 0.94$) (Gillon et al. 1999) and even organic layers in forest soils ($R^2 = 0.96$) (Chodak et al. 2002) have shown a similar accuracy as the calibration model developed in this study. Our results, in conjunction with these studies, illustrate the effectiveness of NIRS to predict nitrogen content of tissue regardless of the tissue type.

Despite the lower coefficient of determination value of our carbon model ($R^2 = 0.84$) relative to our nitrogen and phlorotannin models, the carbon model still exhibited high predictive power when tested against the validation set ($R^2 = 0.95$). The lower value could be due to the small range of the carbon values (25%–28% dry weight) in the calibration set. Gillon et al. (1999) found a similarly variable relationship ($R^2 = 0.86$) when measuring the carbon content of senescent pine needles that ranged ~49%–54% dry weight. However, when Gillon et al.

TABLE 3. Repeated measures analysis of variance (ANOVA) test statistics (degrees of freedom, *F* and *P*) for differences in phlorotannin content (phloroglucinol equivalents % dry weight), nitrogen content (% dry weight), carbon content (% dry weight), and C:N ratio between new and old *Sargassum* tissue and the effects of temperature (*T*) and ammonium enrichment level (N).

	Response variables											
	Phlorotannin			Nitrogen			Carbon			C:N ratio		
Effects	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Between												
Temperature (*T*)	1	0.14	0.717	1	14.12	*<0.001*	1	0.02	0.888	1	16.12	*<0.001*
Ammonium (N)	3	3.26	*0.040*	3	3.76	*0.024*	3	20.80	*<0.001*	3	8.12	*<0.001*
$T \times$ N	3	0.39	0.763	3	0.60	0.624	3	0.78	0.518	3	0.56	0.648
Error	23			24			24			24		
Within												
Age	1	0.73	0.402	1	62.22	*<0.001*	1	16.12	*<0.001*	1	102.06	*<0.001*
Age $\times$ *T*	1	1.17	0.292	1	0.00	0.980	1	0.57	0.458	1	2.60	0.120
Age $\times$ N	3	0.18	0.907	3	1.35	0.281	3	2.01	0.140	3	1.43	0.259
Age $\times$ *T* $\times$ N	3	3.12	*0.046*	3	1.51	0.237	3	0.19	0.902	3	1.44	0.255
Error	23			24			24			24		

Significant values ($P < 0.05$) are given in italics.

(1999) increased the range of carbon in the calibration set to ~32%–54% dry weight by adding green pine needles and leaf litter to the calibration set, the coefficient of determination of the NIRS model increased to $R^2 = 0.99$.

Using NIRS to measure variation in plant secondary metabolite concentrations. We aimed to determine if an appropriate NIRS model could be developed to measure phlorotannin content (as phloroglucinol equivalents) in the brown alga *S. flavicans* as an alternative to traditional wet chemistry methods to aid in algal studies using small tissue samples. The high predictive power ($R^2 = 0.91$) of the phlorotannin model developed in this study demonstrates that NIRS is an accurate alternative method to quantify phlorotannins in *Sargassum*.

Until now, studies investigating secondary metabolites in algae have relied on colorimetric or HPLC methods. Although the precision of NIRS predictions can never be higher than the initial data used to calibrate the models (in this case colorimetric data), the use of NIRS provides valuable advantages over traditional methods. Although the initial outlay cost for the instrument is high, the benefits of using NIRS to measure algal secondary chemistry over traditional methods include reduced processing time (samples only need to be dried and ground), the nonconsumptive nature of analysis (allowing tissue to be used in further experiments), the small sample size required (as little as ~0.15 g), the ability to simultaneously measure different constituents on the same sample, and the reduced costs of laboratory consumables.

In terrestrial studies, NIRS has already been used to measure secondary metabolites with known effects on herbivores and on wider ecosystem processes. Couteaux et al. (2005) used NIRS successfully to determine the water-soluble and total extractable polyphenolics of forbs, grasses, shrubs, and giant rosettes from not only different organs (leaves, stems, roots) but also at different decomposition stages, demonstrating the versatility of NIRS to measure secondary metabolites from a diverse range of plant tissues. Additionally, Henery et al. (2008) developed NIRS models to quantify formylated phloroglucinol compounds in *Eucalyptus* trees, which have been proposed to act as defensive compounds against insect herbivores. The compound specificity of the NIRS models in Henery et al. (2008) suggests that it will be possible to further develop NIRS models to target specific secondary compounds in algae. Due to the laboratory facilities and time required to carry out more compound-specific analyses of secondary metabolites in algae, many ecological studies adhere to the crude analyses of total groups of compounds, as was done in this study using the Folin–Ciocalteus method. The development of NIRS models that predict more specific compounds could further enhance the scope of many algal chemical ecology studies for a number of reasons. The reduced cost (after initial outlay), time, and sample required by NIRS to measure specific secondary compounds would allow for high levels of replication in algal studies. Macroalgae frequently display high levels of variation in secondary metabolite production (e.g., among populations, individuals, and tissue types; Van Alstyne et al. 1999, 2001), and higher replication would allow greater detection of patterns of secondary metabolite production in response to treatments/variables above this background variation.

The small amount of tissue needed for NIRS analysis would allow for easier determination of small-scale patterns in the spatial distribution of secondary metabolites among algal tissues. The concentrations of secondary metabolites, including phlorotannins, can vary among tissues on small scales (<1 cm), and it has been shown that small marine herbivores (such as the amphipods and isopods collectively termed mesograzers) are able to

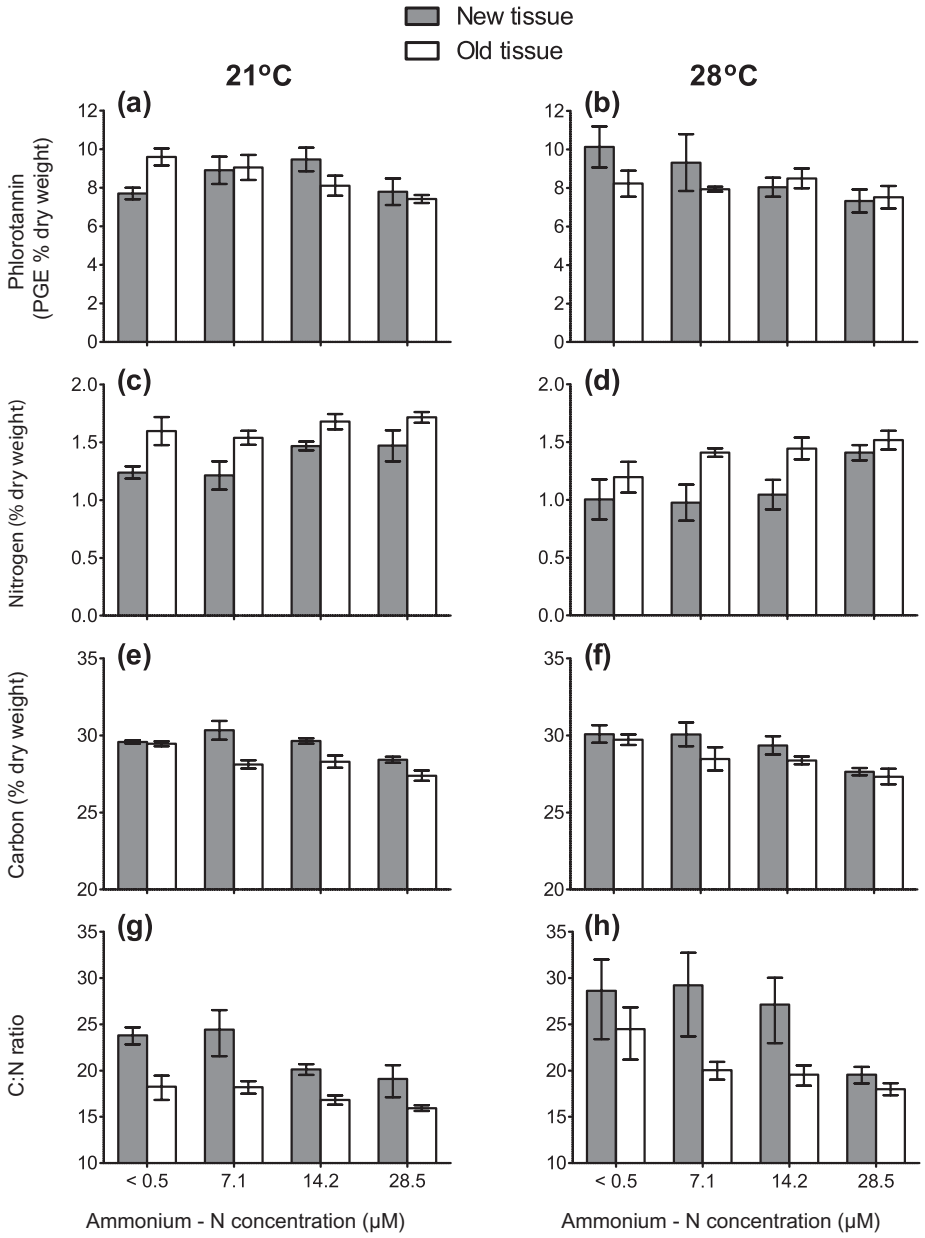

FIG. 4. Mean ± SE chemical composition of *Sargassum flavicans* tissue grown at 21°C (a, c, e, and g) and 28°C (b, d, f, and h). Tissue phlorotannin content (phloroglucinol equivalents % dry weight) (a and b), nitrogen content (% dry weight) (c and d), carbon content (% dry weight) (e and f), and C:N ratio (g and h). Dark bars represent new tissue, and open bars represent older tissue. PGE% = phloroglucinol equivalents of dry weight.

select among tissues on these scales (Poore 1994). As marine macroalgae are exposed to a diverse range of mesograzers, the ability to establish how secondary metabolites are affecting herbivore foraging patterns, and how localized induction of metabolites as a response to damage (Hemmi et al. 2004) may protect algae, will depend on measuring metabolite concentrations on the scales relevant to the herbivore. Similarly, the within-individual distribution of secondary metabolites has important effects on their ability to deter fouling organisms from algal tissues (Steinberg et al. 2001).

Using NIRS to measure how plants respond to environmental change. Plant traits are affected by, and respond to, environmental change (Chapin 2003). In coastal areas, assessment of algal traits is particularly important to identify how algal communities as a whole are affected by eutrophication of coastal waters. Effectively determining the impacts of nutrient enrichment on the primary producers of a system is critical to highlight possible nutrient-induced changes to higher trophic levels in coastal ecosystems. Effective methods to determine how algal traits respond to climate change will also help us understand the possible consequences of these predicted changes to the coastal ecosystems as a whole.

Using the NIRS models developed in this study, we were able to detect differences in the N, C, and phlorotannin content of *S. flavicans* across experimental treatments of enriched nutrient supply and enhanced temperatures. As predicted, phlorotannin content decreased with elevated nitrogen levels in

conjunction with increased growth rates and declines in C:N of tissue. This inverse relationship between nitrogen concentration of tissue and phlorotannin has previously been described for a range of brown algal species and is proposed to be due to greater allocation to growth rather than carbon-rich secondary metabolites. In the temperate brown alga *Fucus vesiculosus*, phlorotannins have been shown to decrease in response to elevated nitrogen levels (Ilvessalo and Tuomi 1989, Yates and Peckol 1993, Pavia and Toth 2000, Hemmi et al. 2004, Koivikko et al. 2005). Variation in polyphenolic content in the tropical brown alga *Lobophora variegata* also varies as a function of nitrogen availability, decreasing with increasing nitrogen concentrations (Arnold et al. 1995). In contrast, variation in nitrogen did not result in variation in phlorotannin concentrations in *Fucus vesiculosus* (Hemmi et al. 2005), *Ascophyllum nodosum* (Pavia and Toth 2000, Svensson et al. 2007), or *Sargassum filipendula* (Cronin and Hay 1996). Although enriched nitrogen was correlated with decreased phlorotannin in our study, we did observe that both temperature and tissue age modified the effects of nitrogen on tissue phlorotannin (Table 3). This finding indicates that growth conditions and tissue age could partially explain contradictory results found in the literature.

We predicted that increased temperature would result in increased growth and a decline in phlorotannin content as carbon demand for growth would decrease carbon allocation to secondary metabolites. In contrast to our predictions, there was no effect of temperature on growth or phlorotannin content in our study. We also determined that tissue phlorotannin content did not vary with age of tissue despite previous studies of temperate (Connan et al. 2006) and tropical (Stiger et al. 2004) *Sargassum* species that found phlorotannin concentrations were affected by tissue age. This study did, however, show a significant interaction effect between temperature, nutrients, and tissue age on phlorotannin content, illustrating the importance of investigating all three variables in this and potentially other systems. Many previous studies have looked at the interactive effects of nutrients, light, herbivory (simulated and real), and tissue type on phlorotannin content (Yates and Peckol 1993, Cronin and Hay 1996, Hammerstrom et al. 1998, Pavia and Toth 2000, Pavia et al. 2003, Hemmi et al. 2004, 2005, Koivikko et al. 2005, Edwards et al. 2006, Fairhead et al. 2006, Svensson et al. 2007). This study suggests that temperature may be another important factor regulating phlorotannin production under some environmental conditions, and further work should be conducted in this area, particularly in view of enhanced sea temperatures associated with global climate change.

In conclusion, given its analytical speed and accuracy, NIRS can be a valuable tool for measuring the tissue traits of nitrogen, carbon, and phlorotannin in algal studies. In particular, NIRS has the potential to enhance studies of algal-herbivore interactions via its ability to measure multiple constituents from small tissue samples without consuming those samples, allowing their use in further analyses or experiments.

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