LIMNOLOGY
and
OCEANOGRAPHY: METHODS

ASLO

Limnol. Oceanogr.: Methods 13, 2015, 9–14

doi: 10.1002/lom3.10002

Testing sample stability using four storage methods and the macroalgae *Ulva* and *Gracilaria*

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Abstract

Concern over the relative importance of different sample preparation and storage techniques frequently used in stable isotope analysis of particulate nitrogen (δ^{15}N) and carbon (δ^{13}C) prompted an experiment to determine how important such factors were to measured values in marine organisms. We stored the marine macroalgae *Ulva* and *Gracilaria* in four different ways and analyzed replicates every three months over the course of a year to assess treatment effects on stability. Treatments consisted of algae dried at 65°C, ground to a powder, and stored in a desiccator until analysis; algae left in a drying oven or in a freezer and processed (dried and ground) just prior to analysis, as well as some dried, ground samples kept out in the lab and reanalyzed quarterly for 12 months. Concurrently, to assess the ecological range in isotope values over the course of a year, samples were freshly collected from the same location and analyzed along with the other treatments at each time step. Neither storage technique nor time had an impact on either δ^{15}N or δ^{13}C values or the %N and %C of the algae tissues. There were clear and consistent differences between species and some large seasonal differences in the freshly collected samples. The interspecies differences and seasonal ranges of values underscore the stability associated with method and duration of sample storage.

Oftentimes laboratory procedures, like legends, are passed down from one analyst to the next, as previous experiences have determined the methods necessary to obtain the best results. However, sometimes the reasoning behind these methods is lost and a reassessment is needed. In using stable isotopes of nitrogen (δ^{15}N) and carbon (δ^{13}C) in our own work, we have followed procedures developed by colleagues as well as adopted practices described in the literature. As ecologists, we frequently collect plant and animal tissues, as well as sediment, from coastal areas which are then cleaned with deionized water, dried in a 65°C oven, ground to a powder, and then analyzed on an isotope ratio mass spectrometer. Although the paradigm has always been to analyze the samples quickly after collection, it has not always been feasible. Although taught to store samples in a desiccator prior to analysis, the sheer number of samples has precluded this practice for all samples. From issues like these arose concern about the stability of the samples with respect to storage time and method. We conducted an experiment to test the stability of samples of macroalgae commonly found in our region (Southern New England, *Ulva* and *Gracilaria*) over the course of a year, under four different storage methods.

Typically, published methods call for samples to be dried in an oven (~60°C) for 24 h or until dry (Wozniak et al. 2006; Oczkowski et al. 2008). But, it is unclear whether samples can be dried for "too long," where extensive exposure to heat (days, weeks, or months) would eventually enhance tissue breakdown and alter results. In addition to examining the effects of dried, ground samples left in a desiccator and on the benchtop (in sealed scintillation vials), we included a drying treatment where samples were left in open aluminum weigh pans in a drying oven for up to one year. Finally, to approximate a fresh sample, subsamples were frozen and individually defrosted, dried, and ground within a week of analysis. To assess stability over time, some subsamples were

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analyzed after our initial collection and then periodically over the course of a year. If sample degradation were to occur, we could observe an increase in isotope value over time as the lighter isotope might be preferentially lost (e.g., Fry 2006). We further hypothesized that samples left on the counter might contain more water compared to those in a desiccator, which could both facilitate the decomposition of the sample and possibly distort the masses weighed for individual sample analyses, thus distorting the measured %N and %C values. Also, if the long-term heat of the drying oven aided in the breakdown and volatilization of N, we might expect to see a change in the δ^{15}N values and a decrease in the %N. Our results (thankfully) indicate that the isotope and N and C contents of the two macroalgae genera examined were stable over time and among treatments. Given the range of ecological data, sample storage technique may have an inconsequential impact on analytical outcome.

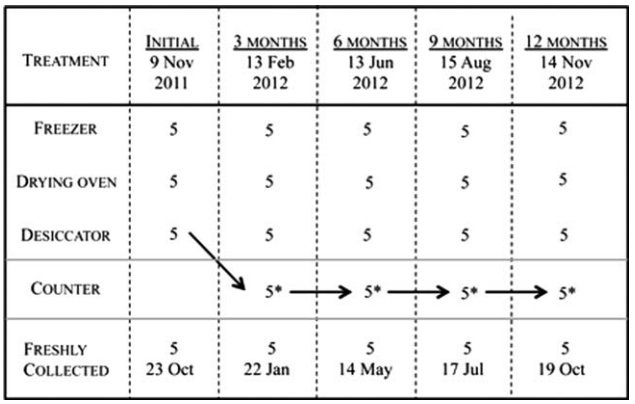

Fig. 1. Schematic of treatments for each species. For freezer, drying oven, and desiccator treatments, 75 total individuals were collected in Fall 2011, and 15 were analyzed at each time point (five per treatment). For the freshly collected samples, five specimens were collected from the field at each time point. Dates listed indicate mass spectrometer run dates. *Indicates repeated analysis on same samples ("counter" treatment).

Materials and Procedures

Sample collection and processing

We collected 75 samples each of *Ulva rigida* C. Agardh and *Gracilaria vermiculophylla* (Ohmi) Papenfuss from Oakland Beach, RI (41.68399, −71.39787) on 23 October 2011. All algal thalli (individuals) were brought back to the lab and immediately sorted to the species level, obvious epiphytes were removed, and algae were rinsed with deionized water. Samples were allocated as follows for *Ulva* and *Gracilaria*: 20 individuals of each species were cleaned, placed in sealed zipper bags, and placed into a freezer (−20°C) until later analysis (hereafter "freezer" samples; *see* Fig. 1 for sample breakdown). The remaining 55 individuals of each species were cleaned, placed into separate aluminum weighing dishes, and then into a drying oven at 60°C. Once these were dry (after two days), 15 were promptly removed, ground individually into a fine powder with a mortar and pestle, and 2 mg to 3 mg of tissue from each sample were placed into individual capsules for mass spectrometry analysis (9 Nov 2011). These fifteen specimens were randomly allocated as the initial samples for one of three storage treatments (five for freezer, five for drying oven, and five for desiccator) for *Ulva* and *Gracilaria* (Fig. 1). In addition to serving as "initial" data points for the different treatments, the five initial desiccator samples were left out on the counter and reanalyzed at each subsequent time step. Although this allowed us to look for changes over time in samples stored on the counter, they were treated separately in statistical analyses (as described below).

For each species, the remaining forty samples were divided into two equally sized treatments named "desiccator" and "drying oven." Desiccator samples were removed from the drying oven, immediately ground into powder, and stored in 20 scintillation vials in a laboratory desiccator. Drying oven samples remained as intact thalli in the drying oven. At set time points (February, June, August, and November 2012—based in part on mass spectrometer availability), we removed five individuals from each of the three treatments, for each species, and analyzed them in a mass spectrometer. Prior to analysis, frozen specimens were dried and ground and drying oven specimens were ground. At each subsequent time step at approximately three-month intervals (22 January 2012,14 May 2012,17 July 2012, and 19 October 2012), we collected five fresh individuals from each species from Oakland Beach, cleaned them in the lab, and then dried, ground, and analyzed them (hereafter "freshly collected").

To address some questions that arose regarding initial δ^{15}N isotope values, we collected five additional *Ulva* and *Gracilaria* samples (hereafter called addendum samples) on 13 July 2013 and analyzed them first on 31 July 2013 and then again 23 September 2013. As described above, samples were dried, ground, and stored in acid-washed scintillation vials on the counter until initial and then final analysis for δ^{15}N values.

Sample analysis

Samples were weighed into small tin capsules and analyzed on an Isoprime 100 mass spectrometer interfaced with a Micro Vario Elemental Analyzer (Elementar Americas, Mt. Laurel, NJ) for δ^{15}N, %N, δ^{13}C, and %C. The nitrogen isotope composition was expressed as a part per thousand (permil, ‰) deviation from air while the carbon was referenced to PeeDee Belemnite where $\delta X = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3$, X is δ^{15}N or δ^{13}C, and R is the ratio of heavy to light isotope (^{15}N : ^{14}N, ^{13}C : ^{12}C). Samples were analyzed in triplicate and in batches of approximately 30 samples. Internal standards were used for check for instrument drift

Table 1. Results from three way fixed factor ANOVAs for δ^{15}N, %N, δ^{13}C, %C for frozen, oven, and desiccator samples of *Ulva* and *Gracilaria* and from two way fixed factor ANOVAs from freshly collected samples.

		δ^{15}N			%N			δ^{13}C			%C		
Source	**DF**	**SS**	***F***	***p***	**SS**	***F***	***p***	**SS**	***F***	***p***	**SS**	***F***	***p***
Frozen, oven, and desiccator samples													
Species	1	153.66	153.66	**<0.0001**	13.5	233.77	**<0.0001**	264.41	153.37	**<0.0001**	209.74	86.73	**<0.0001**
Treatment	2	0.34	0.45	0.64	0.03	0.3	0.74	2.92	0.85	0.43	1.85	0.38	0.68
Species × treatment	2	0.64	0.85	0.43	0.03	0.24	0.78	9.35	2.71	0.07	1.01	0.21	0.81
Time	4	26.94	17.89	**<0.0001**	0.67	2.89	**0.02**	15.08	2.19	0.07	18.62	1.92	0.11
Species × time	4	7.28	4.84	0.001	0.58	2.52	**0.04**	13.2	1.91	0.11	34.27	3.54	**0.01**
Treatment × time	8	4.38	1.45	0.18	0.68	1.48	0.17	7.09	0.51	0.84	46.59	2.41	**0.02**
Species × treatment × time	8	2.64	0.87	0.54	0.48	1.05	0.4	19.65	1.42	0.19	27.26	1.41	0.2
Error	120	45.18			6.94			206.88			290.19		
Freshly collected samples													
Species	1	0.04	0.2	0.66	8.67	89.39	**<0.0001**	16.62	5.66	**0.024**	303.71	122.41	**<0.0001**
Time	3	42.58	62.32	**<0.0001**	50.64	173.94	**<0.0001**	123.83	14.07	**<0.0001**	61.02	8.2	**0.0004**
Species × time	3	4.81	7.04	**0.001**	2.42	8.31	**0.0003**	130.08	14.78	**<0.0001**	108.1	14.52	**<0.0001**
Error	31	7.06			3.01			90.96			76.91		

Statistically significant differences are indicated by bold *p*-values.

in each run and to correct for instrument offset. The %N and %C was calculated by comparing the peak area of the unknown sample to a standard curve of peak area vs. standard %N or %C content.

Statistics

We analyzed the changes among treatments, between species, and across time in δ^{15}N, %N, δ^{13}C, and %C of desiccator, drying oven, and freezer samples via a three-way fixed factor ANOVA using JMP v11 statistical software (www.jmp.com). We analyzed changes in the same four parameters for the freshly collected samples between species and across time via a two-way fixed factor ANOVA. Changes in the "counter" samples over time and between species were analyzed with two-way repeated measures ANOVA (using 3, 6, 9, and 12 month data). Addendum samples were analyzed similarly for δ^{15}N with repeated measures ANOVA (using initial and two month data). All data were checked for normality and homogeneity of variances and transformed where appropriate.

Assessment

$\delta^{15}N$

The δ^{15}N values for the oven, desiccator, and freezer samples were, on average 2‰ lower in *Ulva* than *Gracilaria* (Table 1; $F_{1,120} = 153.66$, $p < 0.001$; Fig. 2). However, there were no significant differences in δ^{15}N values across treatments (Table 1; $F_{2,120} = 0.45$, $p = 0.64$). Surprisingly, it does not seem to matter if macroalgae are left uncovered in a drying oven, dried and ground in a desiccator, or in a freezer, prior to analysis, at least in a southern New England climate. We found similar isotope values for samples dried, ground, and left on a counter (Fig. 2). Because the counter samples were reanalyzed repeatedly using material from the same vial, they could not be treated with the same statistical techniques as the drying oven, desiccator, and freezer treatments. Despite the statistical limitations in our ability to directly compare the counter samples to the other treatments, they do not appear distinct from the others.

There was, however, a statistically significant difference in δ^{15}N among analysis dates ($F_{1,120} = 17.89$; $p < 0.0001$) for the oven, desiccator, and freezer samples. Due to the lack of a significant treatment main effect or interactions, we removed treatment from the analyses and re-ran the δ^{15}N analyses separately for each species (as there was a significant species by time interaction). We used time as the main effect to determine which analysis dates differed (Underwood 1997). For *Gracilaria*, δ^{15}N values for the initial samples were significantly higher than those measured at 3, 6, 9, and 12 months ($p < 0.05$). The initial *Ulva* samples were not statistically different from the later measurements ($p = 0.15$). Our "counter" samples did not exhibit significant variability in δ^{15}N across the study period (3 to 12 months; $F_{1.6,\ 13.2} = 3.78$; $p = 0.06$).

The higher initial *Gracilaria* and slightly, but not statistically, higher *Ulva* values may be reflecting some instrument instability during the initial (Nov 2011) measurements of δ^{15}N. As part of our analysis, we used a series of check standards (a homogenized blue mussel tissue that is periodically internally calibrated to standard reference material) interspersed throughout the run. These standards are used to calibrate the reference gas and to check for any instrument drift. Typically, standard deviations around these check standards are well below 0.3‰ and generally <0.2‰. In our initial sampling, the check standards had an average value of

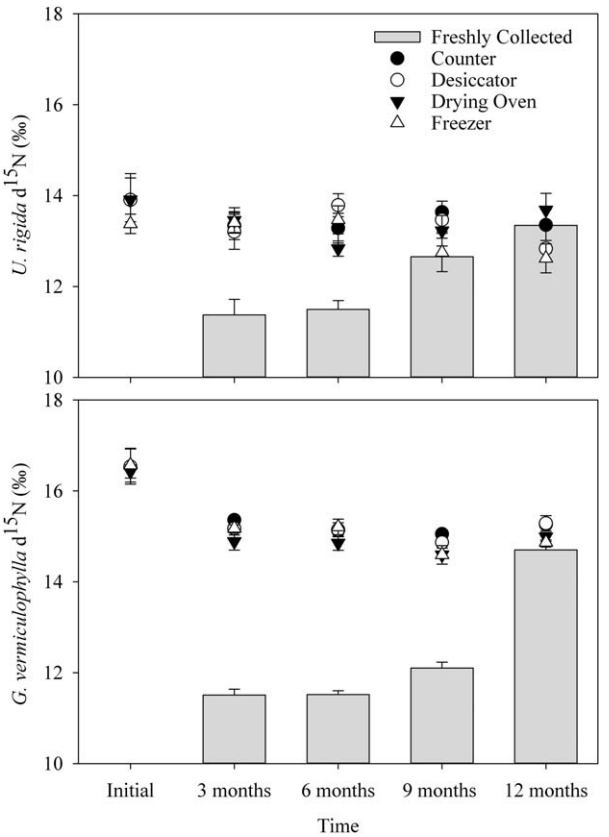

Fig. 2. Mean (±1 standard error) δ^{15}N values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Shapes represent storage techniques (desiccator, drying oven, freezer, and counter) where counter samples were left on the benchtop and periodically reanalyzed, with separate replicates of desiccator, drying oven, and freezer samples that were analyzed at each time step. Bars represent samples freshly collected from the same location just prior to analysis and were included to illustrate the range of values observed seasonally.

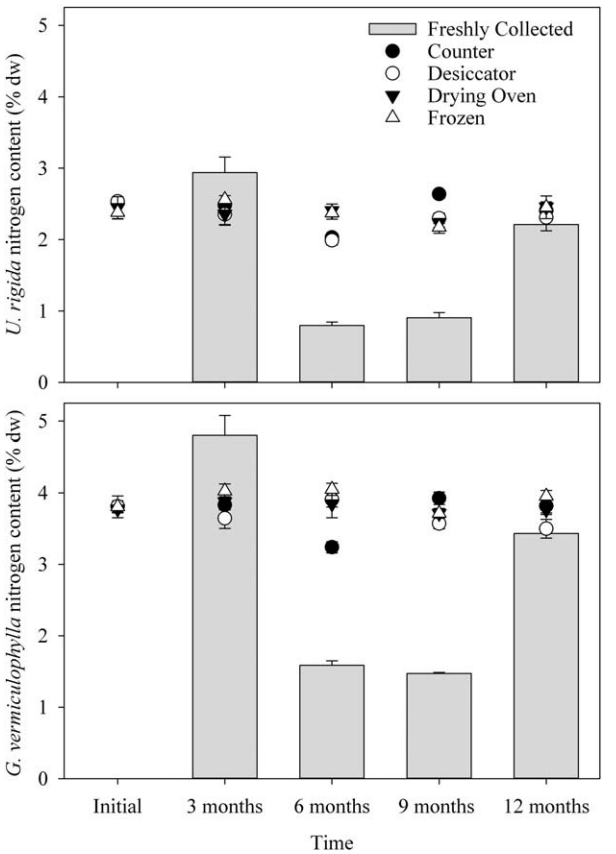

Fig. 3. Mean (±1 standard error) of %N values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

11.68‰ ± 0.64‰ (S.D.). However, the cystine standard that we use to calibrate our %N measurements had a high reproducibility (9.66‰ ± 0.054‰ S.D., n = 4) and the offset between the cystine δ^{15}N values measured in this run and the actual (calibrated to reference material) was the same as for the blue mussel check standard, lending strength to the check standard. But, overall, variability appeared to be higher in this initial run. To address this drop in δ^{15}N values between initial and subsequent sampling, we collected additional samples in July 2013 and analyzed them two weeks and then 10 weeks after collection. The δ^{15}N values in what we termed the addendum samples did not change significantly over time ($F_{1,8}$ = 0.70, p = 0.43), lending support to our supposition that the originally higher initial *Gracilaria* δ^{15}N values were due to instrument performance.

By contrast, there were clear seasonal differences in δ^{15}N in freshly collected macroalgae. With a range of 2‰ for *Ulva* and 4‰ for *Gracilaria*, the highest values were in the late fall and lowest in the winter and spring ($F_{3,31}$ = 62.32, p < 0.0001; Table 1), with a significant interaction ($F_{3,31}$ = 7.04, p = 0.001, Fig. 2), although there was no difference between species ($F_{1,31}$ = 0.20, p = 0.66). The wide range in the values of freshly collected algae underscores the stability of the algae collected initially (23 October 2011), regardless of storage technique.

%N

As with δ^{15}N, there were no significant differences in %N among frozen, oven, and desiccator treatments (Table 1; $F_{2,120}$ = 0.30, p = 0.74; Fig. 3), although %N was significantly higher in *Gracilaria* ($F_{1,120}$ = 233.77; p < 0.0001) and varied significantly among sampling dates ($F_{4,120}$ = 2.89, p = 0.0252). However, when we removed all treatment terms and re-ran the analyses (as for δ^{15}N above), post hoc comparisons did not yield any dates that significantly differed in %N. By contrast, *Gracilaria* left on the counter varied significantly among analysis dates ($F_{1.6,\ 12.69}$ = 101.52, p < 0.0001; Fig. 3), although there was not a consistent trend over time. The lowest values (at six months) may have been associated with samples which were weighed to one less decimal place

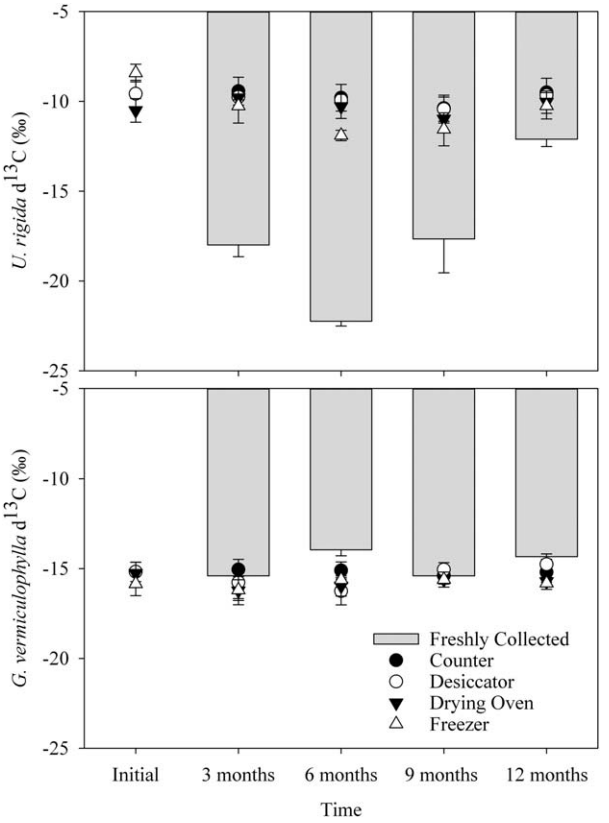

Fig. 4. Mean (±1 standard error) of δ^{13}C values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

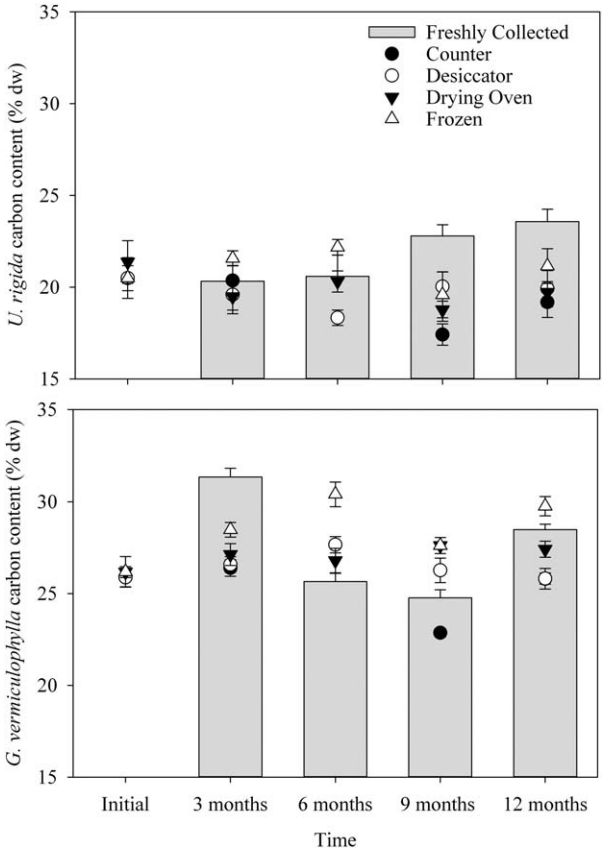

Fig. 5. Mean (±1 standard error) of %C values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

than usual, increasing the uncertainty of the %N (and %C) values.

Overall, *Gracilaria* had about a third more N in their tissues than did *Ulva* (~3.75% vs. ~2.5%, $p < 0.0001$; Table 1; Fig. 3). A recent assessment of *Ulva* and *Gracilaria* in Narragansett Bay found %N ranging from 1% to 5%, with differences in newly formed vs. mature tissues (Thornber et al. 2008). By contrast, our %N values are lower than reported in some other areas for both species (e.g., Abreu et al. 2011; Barr et al. 2013). The freshly collected samples showed a distinct seasonal pattern, where %N was lowest in the spring and summer and highest in the fall and winter months ($F_{3,31} = 173.94$, $p < 0.0001$; Table 1, Fig. 3). Although we suspect these values may be reflecting spring and summer water column nutrient depletion and winter luxury uptake, they nonetheless indicate a dynamic environment.

$\delta^{13}C$

We did not find significant differences in the δ^{13}C content of algae among oven, freezer, desiccator treatments, or among analytical dates (Table 1), although *Ulva* had much higher δ^{13}C values (~ −10‰) than *Gracilaria* (~ −15 ‰; $F_{1,120} = 153.37$, $p < 0.0001$; Fig. 4). By contrast, we did find significant differences in δ^{13}C in our counter specimens that were repeatedly sampled ($F_{1.3,10.8} = 37.46$, $p < 0.0001$; Fig. 4), with a significant time by species interaction ($F_{1.3,10.8} = 11.79$, $p = 0.004$).

There has been substantial detailed work in cataloging and interpreting differences in C isotopes among species, as these values can be indicative of how the species acquire C from the environment as well as their photosynthetic performance (for example, *see* Fry and Sherr 1984; Raven et al. 1995, 2002). Although these discussions are beyond the scope of this article, it is useful to note that our measured values indicate that these species are capable of taking up both CO_2 and HCO_3^- although isotope differences between the two forms of inorganic carbon does not indicate proportional uptake of either carbonate species (Raven et al. 2002). While variable, other measurements of δ^{13}C values from macroalgae in Narragansett Bay have ranged from −26‰ to −12‰ (Oczkowski et al. 2008). And, our freshly collected *Ulva* samples similarly ranged from −22.23‰ to −9.5‰ throughout the year. In contrast, *Gracilaria* was more homogenous, with mean values ranging only from −15.43‰ to −13.96‰; values were significantly higher (less negative) for *Gracilaria* than *Ulva* ($F_{1,31} = 5.67$, $p = 0.24$; Table 1),

with significant variation among sampling dates ($p < 0.0001$) and a significant species by time interaction ($p < 0.0001$). Overall, although our measured *Gracilaria* values are typical for this region, *Ulva* values from the initial (October 2011) collection were slightly higher than previously measured, but not uncharacteristically so for macroalgae (Raven et al. 2002; Oczkowski et al. 2008).

%C

As with the other parameters measured, the %C of the macroalgae (23.8 ± 0.33%) did not vary significantly among freezer, oven, and desiccator treatments ($F_{2,120} = 0.38$, $p = 0.68$; Table 1; Fig. 5) nor over analysis dates ($F_{4,120} = 1.92$, $p = 0.11$), although the %C was significantly higher in *Gracilaria* than *Ulva* (27.3% vs. 20.2%; $F_{1,120} = 86.73$, $p < 0.0001$). The %C of the freshly collected samples was significantly higher in *Gracilaria* than *Ulva* ($p < 0.0001$, Table 1), where %C of *Gracilaria* ranged from 24.76% to 31.35% and *Ulva* from 20.33% to 23.57%. Samples from January were the highest, followed by samples from October 2012, and then May and July 2012 ($p < 0.0001$, Table 1, Tukey post hoc comparisons).

Discussion

We chose to conduct an experiment to assess sample stability using several common sample storage techniques. Using macroalgae, our results clearly indicate that sample storage method has no bearing on the resultant δ^{15}N, %N, δ^{13}C, and %C values. This is particularly surprising for those samples left in open weighing tins in a 65°C drying oven for up to a year prior to analysis. We speculate that these results are transferrable to many other plant tissues and maybe even to some animal tissues as well.

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Acknowledgments

Thank you to R. McKinney for assistance with sample analysis, and to J. Carey, R. Johnson, and M. Pelletier for providing useful edits of this manuscript. Partial funding was provided by the University of Rhode Island through an undergraduate research grant to A. Ziegler. This material is based, in part, upon work supported in part by the National Science Foundation EPSCoR Cooperative Agreement #EPS-1004057 and the State of Rhode Island. This is ORD Tracking Number ORD-008193 of the Atlantic Ecology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U. S. Environmental Protection Agency. Although the research described in this article has been funded in part by the U.S. Environmental Protection Agency, it has not been subjected to Agency review. Therefore, it does not necessarily reflect the views of the Agency.

Submitted 16 May 2014
Revised 6 November 2014
Accepted 1 December 2014

Associate editor: Paul F. Kemp