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Running Head: Stability of macroalgal samples for stable isotope analysis

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47 Abstract

48

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

65

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67 Key words: stable isotope, *Ulva*, *Gracilaria*, nitrogen, carbon

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70 Introduction

71

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

87 Typically, published methods call for samples to be dried in an oven (~60°C) for
88 24 hours or until dry (Oczkowski et al. 2008; Wozniak et al. 2006). But, it is unclear
89 whether samples can be dried for 'too long,' where extensive exposure to heat (days,
90 weeks, or months) would eventually enhance tissue breakdown and alter results. In
91 addition to examining the effects of dried, ground samples left in a desiccator and on the
92 bench-top (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 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 $\delta^{15}N$ 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.

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 October 23, 2011. All algal thalli (individuals) were brought back to the lab and

116 immediately sorted to the species level, obvious epiphytes were removed, and algae were
117 rinsed with deionized water. Samples were allocated as follows for *Ulva* and *Gracilaria*:
118 twenty individuals of each species were cleaned, placed in sealed zipper bags, and placed
119 into a freezer (-20°C) until later analysis (hereafter 'freezer' samples; see Fig. 1 for
120 sample breakdown). The remaining fifty-five individuals of each species were cleaned,
121 placed into separate aluminum weighing dishes, and then into a drying oven at 60°C.
122 Once these were dry (after 2 days), fifteen were promptly removed, ground individually
123 into a fine powder with a mortar and pestle, and 2 to 3 mg of tissue from each sample
124 were placed into individual capsules for mass spectrometry analysis (Nov 9, 2011).
125 These fifteen specimens were randomly allocated as the initial samples for one of three
126 storage treatments (five for freezer, five for drying oven, and five for desiccator) for *Ulva*
127 and *Gracilaria* (Fig. 1). In addition to serving as 'initial' data points for the different
128 treatments, the five initial desiccator samples were left out on the counter and re-analyzed
129 at each subsequent time step. While this allowed us to look for changes over time in
130 samples stored on the counter, they were treated separately in statistical analyses (as
131 described below).
132 For each species, the remaining forty samples were divided into two equally sized
133 treatments named 'desiccator' and 'drying oven'. Desiccator samples were removed from
134 the drying oven, immediately ground into powder, and stored in twenty scintillation vials
135 in a laboratory desiccator. Drying oven samples remained as intact thalli in the drying
136 oven. At set time points (February, June, August, and November 2012 -- based in part on
137 mass spectrometer availability), we removed five individuals from each of the three
138 treatments, for each species, and analyzed them in a mass spectrometer. Prior to analysis,

139 frozen specimens were dried and ground, and drying oven specimens were ground. At
140 each subsequent time step at approximately three-month intervals (January 22, May 14,
141 July 17, and October 19, 2012), we collected five fresh individuals from each species
142 from Oakland Beach, cleaned them in the lab, and then dried, ground, and analyzed them
143 (hereafter 'freshly collected').

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

150

151 Sample analysis

152 Samples were weighed into small tin capsules and analyzed on an Isoprime 100
153 mass spectrometer interfaced with a Micro Vario Elemental Analyzer (Elementar
154 Americas, Mt. Laurel, NJ) for $\delta^{15}N$, %N, $\delta^{13}C$, and %C. The nitrogen isotope
155 composition was expressed as a part per thousand (permil, ‰) deviation from air, while
156 the carbon was referenced to PeeDee Belemnite (PDB) where $\delta X = [(R_{sample}-R_{standard})/$
157 $R_{standard}] \times 10^3$, X is $\delta^{15}N$ or $\delta^{13}C$, and R is the ratio of heavy to light isotope (^{15}N:^{14}N,
158 ^{13}C:^{12}C). Samples were analyzed in triplicate and in batches of approximately 30
159 samples. Internal standards were used for check for instrument drift in each run and to
160 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 $\delta^{15}N$, %N, $\delta^{13}C$, 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 a two-way repeated measures ANOVA (using 3, 6, 9, and 12 month data). Addendum samples were analyzed similarly for $\delta^{15}N$ with a repeated measures ANOVA (using initial and 2 month data). All data were checked for normality and homogeneity of variances and transformed where appropriate.

Assessment

$\delta^{15}N$

The $\delta^{15}N$ 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 $\delta^{15}N$ 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

184 in a drying oven, dried and ground in a desiccator, or in a freezer, prior to analysis, at
185 least in a southern New England climate. We found similar isotope values for samples
186 dried, ground, and left on a counter (Fig. 2). Because the counter samples were
187 reanalyzed repeatedly using material from the same vial, they could not be treated with
188 the same statistical techniques as the drying oven, desiccator, and freezer treatments.
189 Despite the statistical limitations in our ability to directly compare the counter samples to
190 the other treatments, they do not appear distinct from the others.

191 There was, however, a statistically significant difference in $\delta^{15}N$ among analysis
192 dates ($F_{1,120} = 17.89$; $p<0.0001$) for the oven, desiccator, and freezer samples. Due to the
193 lack of a significant treatment main effect or interactions, we removed treatment from the
194 analyses and re-ran the $\delta^{15}N$ analyses separately for each species (as there was a
195 significant species by time interaction). We used time as the main effect to determine
196 which analysis dates differed (Underwood 1997). For *Gracilaria*, $\delta^{15}N$ values for the
197 initial samples were significantly higher than those measured at 3, 6, 9, and 12 months
198 ($p<0.05$). The initial *Ulva* samples were not statistically different from the later
199 measurements ($p = 0.15$). Our 'counter' samples did not exhibit significant variability in
200 $\delta^{15}N$ across the study period (3 to 12 months; $F_{1.6, 13.2} = 3.78$; $p = 0.06$).

201 The higher initial *Gracilaria* and slightly, but not statistically, higher *Ulva* values
202 may be reflecting some instrument instability during the initial (Nov. 2011)
203 measurements of $\delta^{15}N$. As part of our analysis, we used a series of check standards (a
204 homogenized blue mussel tissue that is periodically internally calibrated to standard
205 reference material) interspersed throughout the run. These standards are used to calibrate
206 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 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 $\delta^{15}N$ 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 $\delta^{15}N$ values between initial and subsequent sampling, we collected additional samples in July 2013 and analyzed them 2 weeks and then 10 weeks after collection. The $\delta^{15}N$ 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* $\delta^{15}N$ values were due to instrument performance.

By contrast, there were clear seasonal differences in $\delta^{15}N$ 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 $\delta^{15}N$, 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 $\delta^{15}N$ 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 6 months) may have been associated with samples which were weighed to one less decimal place 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). While 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 $\delta^{13}C$ content of algae among oven, freezer, desiccator treatments, or among analytical dates (Table 1), although *Ulva* had much higher $\delta^{13}C$ values (~-10 ‰) than *Gracilaria* (~-15 ‰; $F_{1,120} = 153.37$, $p < 0.0001$;

253 Fig. 4). By contrast, we did find significant differences in $\delta^{13}C$ in our counter specimens
254 that were repeatedly sampled ($F_{1.3,10.8} = 37.46$, $p < 0.0001$; Fig. 4), with a significant time
255 by species interaction ($F_{1.3, 10.8} = 11.79$, $p = 0.004$).
256 There has been substantial detailed work in cataloging and interpreting
257 differences in C isotopes among species, as these values can be indicative of how the
258 species acquire C from the environment as well as their photosynthetic performance (for
259 example, see Fry and Sherr 1984; Raven et al. 1995, 2002). While these discussions are
260 beyond the scope of this paper, it is useful to note that our measured values indicate that
261 these species are capable of taking up both CO_2 and HCO_3^- although isotope differences
262 between the two forms of inorganic carbon does not indicate proportional uptake of either
263 carbonate species (Raven et al. 2002). While variable, other measurements of $\delta^{13}C$ values
264 from macroalgae in Narragansett Bay have ranged from -26 to -12 ‰ (Oczkowski et al.
265 2008). And, our freshly collected *Ulva* samples similarly ranged from -22.23 to -9.5‰
266 throughout the year. In contrast, *Gracilaria* was more homogenous, with mean values
267 ranging only from -15.43 to -13.96‰; values were significantly higher (less negative) for
268 *Gracilaria* than *Ulva* ($F_{1,31} = 5.67$, $p = 0.24$; Table 1), with significant variation among
269 sampling dates ($p<0.0001$) and a significant species by time interaction ($p<0.0001$).
270 Overall, while our measured *Gracilaria* values are typical for this region, *Ulva* values
271 from the initial (October 2011) collection were slightly higher than previously measured,
272 but not uncharacteristically so for macroalgae (Raven et al. 2002; Oczkowski et al. 2008).
273
274
275

%C

As with the other parameters measured, the %C of the macroalgae (23.8 $\pm$ 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%C; $F_{1,120} = 86.73$, $p < 0.0001$). 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 $\delta^{15}N$, %N, $\delta^{13}C$, 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.

299 References

300

301 Abreu, M. H., R. Pereira, A. H. Buschmann, I. Sousa-Pinto, and C. Yarish. 2011.
302 Nitrogen uptake responses of *Gracilaria vermiculophylla* (Ohmi) Papenfuss under
303 combined and single addition of nitrate and ammonium. J. Exp. Mar. Biol. Ecol.
304 407**:**190-199.
305 Barr, N. G., B. D. Dudley, K. M. Rogers, and C. D. Cornelisen. 2013. Broad-scale
306 patterns of tissue δ15N and tissue-N indicied in frondose *Ulva* spp.; Developing a
307 national baseline indicator of nitrogen-loading in coastal New Zealand. Mar.
308 Pollut. Bull. 67**:**203-216.
309 Fry, B. 2006. Stable Isotope Ecology. Springer.
310 Fry, B., and E. B. Sherr. 1984. δ13C measurements as indicators of carbon flow in
311 marine and freshwater ecosystems. Contrib. Mar. Sci. 27**:**15-47.
312 Oczkowski, A. J. and others 2008. Distribution and trophic importance of anthropogenic
313 nitrogen in Narragansett Bay: An assessment using stable isotopes. Estuaries and
314 Coasts 31**:**53-69.
315 Raven, J. A. and others 2002. Mechanistic interpretation of carbon isotope discrimination
316 by marine macroalgae and seagrasses. Funct. Plant Biol. 29**:**355-378.
317 Raven, J. A., D. I. Walker, A. M. Johnston, L. L. Handley, and J. E. Kubler. 1995.
318 Implications of 13C natural abundance measurements for photosynthetic
319 performance by marine macrophytes in their natural environment. Mar. Ecol.
320 Prog. Ser. 123**:**193-205.

Thornber, C. S., P. Dimilla, S. W. Nixon, and R. A. Mckinney. 2008. Natural and anthropogenic nitrogen uptake by bloom-forming macroalgae. Mar. Pollut. Bull. 56**:**261-269.

Underwood, A. J. 1997. Experiments in Ecology. Cambridge University Press.

Wozniak, A. S., C. T. Roman, S. C. Wainright, R. A. Mckinney, and M.-J. James-Pirri. 2006. Monitoring food web changes in tide-restored salt marshes: A carbon stable isotope approach. Estuaries and Coasts 29**:**568-578.

344 Figures

345

346

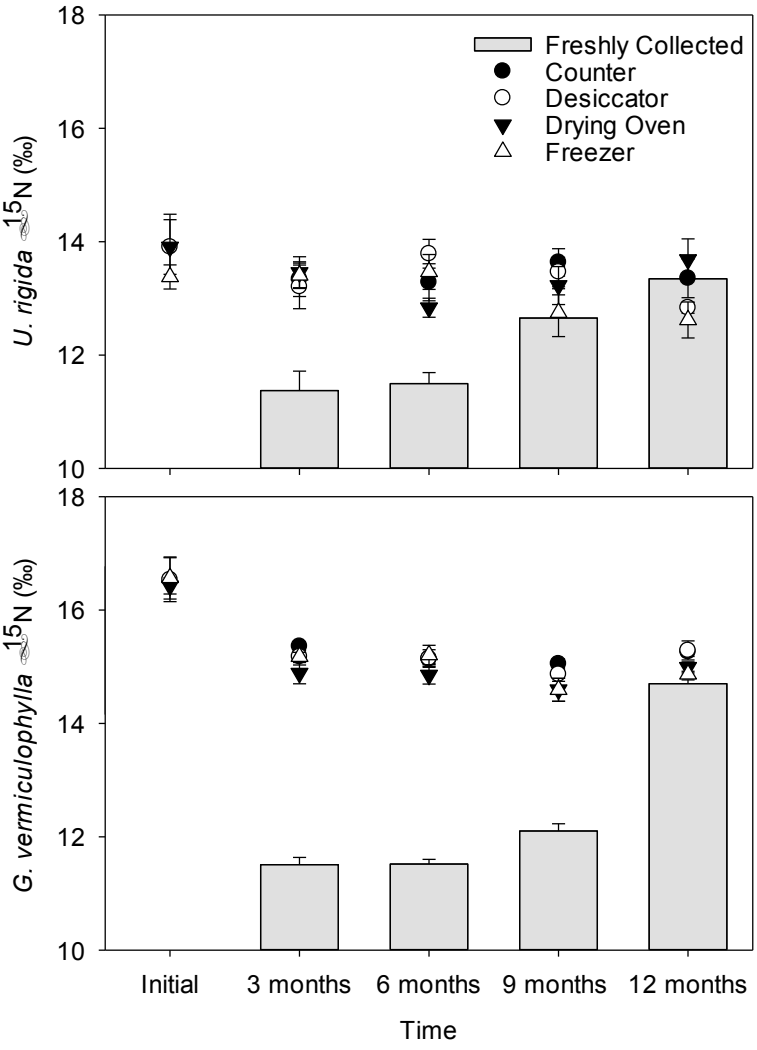
Treatment	Initial 9 Nov 2011	3 months 13 Feb 2012	6 months 13 Jun 2012	9 months 15 Aug 2012	12 months 14 Nov 2012
Freezer	5	5	5	5	5
Drying oven	5	5	5	5	5
Desiccator	5	5	5	5	5
Counter		5* →	5* →	5* →	5*
Freshly Collected	5 23 Oct	5 22 Jan	5 14 May	5 17 Jul	5 19 Oct

347

348

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

355



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358 Figure 2. Mean (± 1 standard error) $\delta^{15}N$ values for *U. rigida* (top panel) and *G.*

359 *vermiculophylla* (bottom panel) over the length of the experiment. Shapes represent

360 storage techniques (desiccator, drying oven, freezer, and counter) where counter samples

361 were left on the bench-top and periodically reanalyzed, with separate replicates of

362 desiccator, drying oven, and freezer samples that were analyzed at each time step. Bars

363 represent samples freshly collected from the same location just prior to analysis and were

364 included to illustrate the range of values observed seasonally.

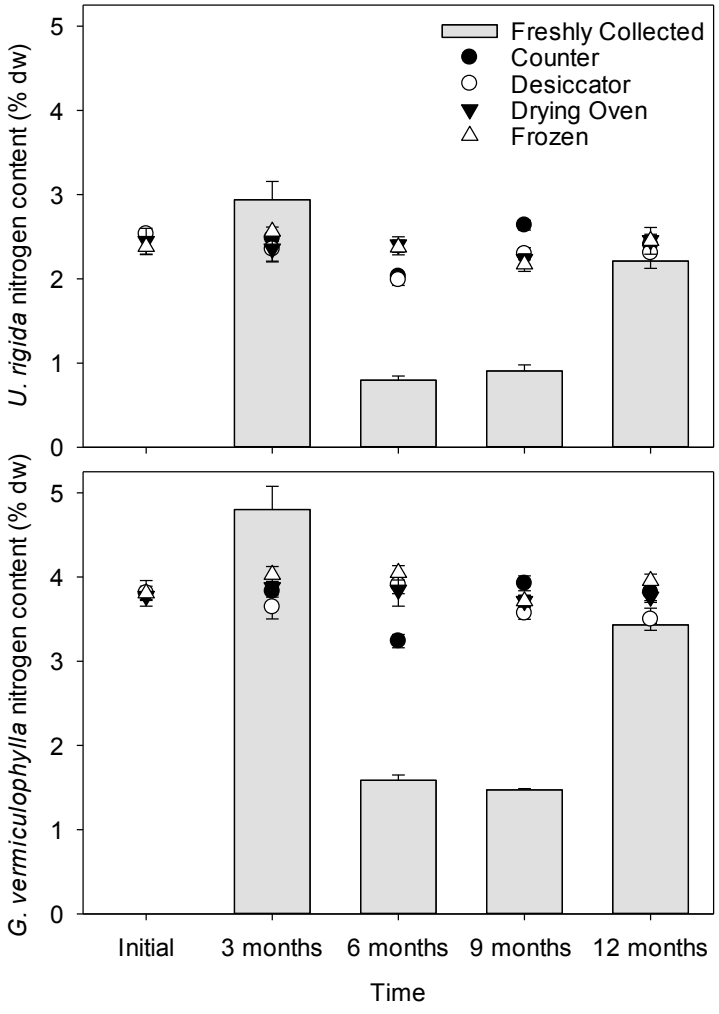

Figure 3. Mean (± 1 standard error) of %N values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

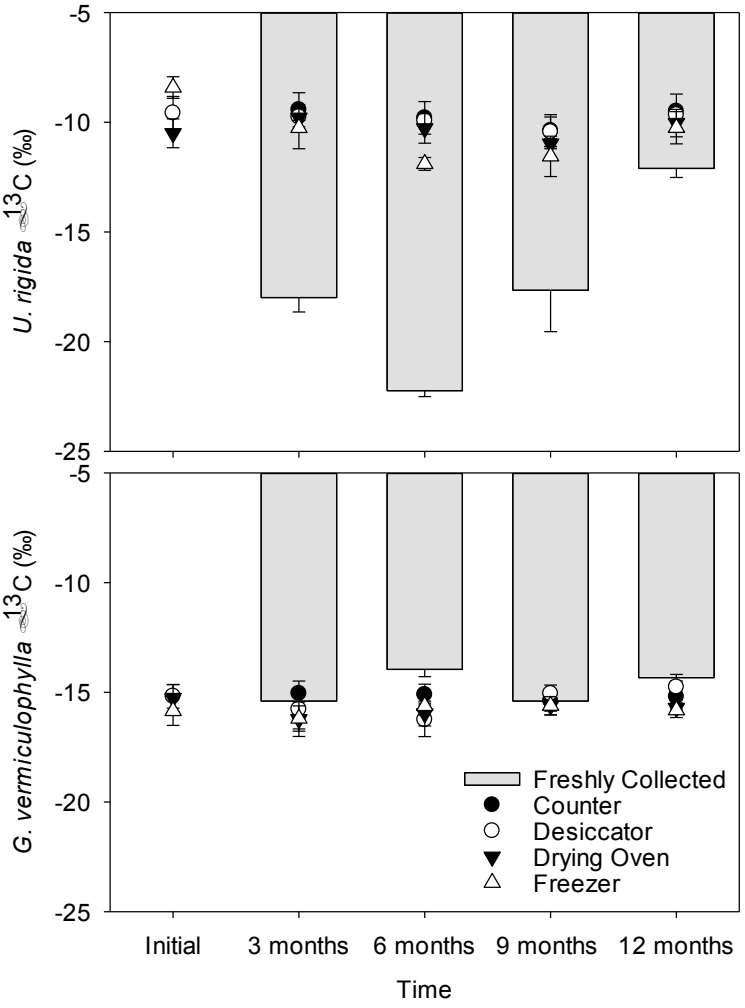

Figure 4. Mean (± 1 standard error) of $\delta^{13}C$ values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

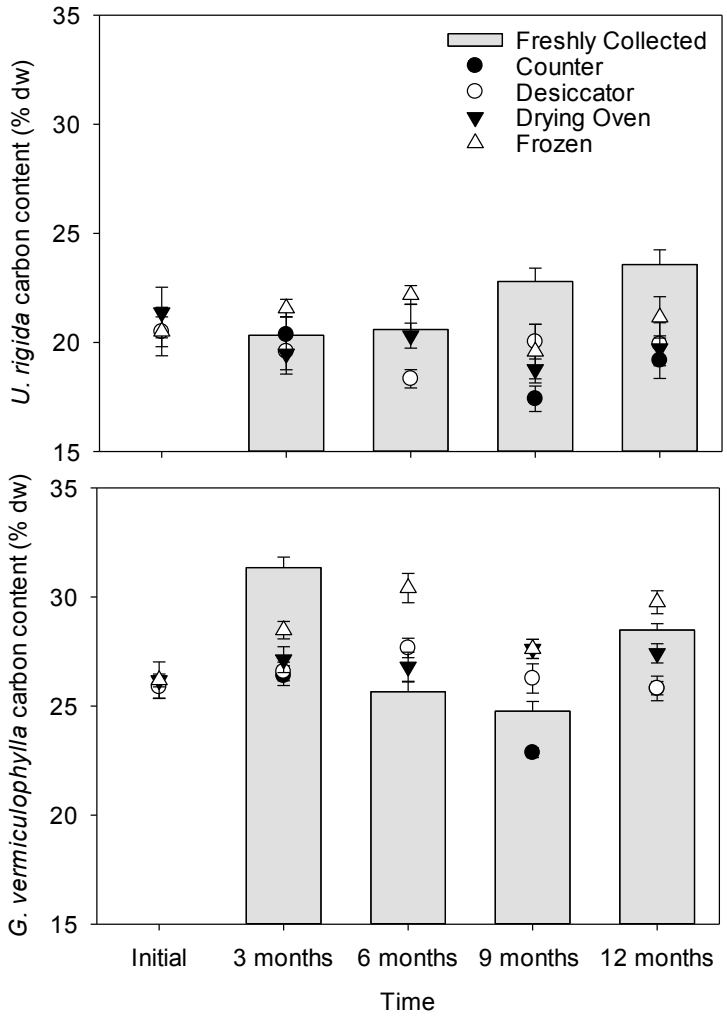

Figure 5. Mean (± 1 standard error) of %C values for *U. rigida* (top panel) and *G. vermiculophylla* (bottom panel) over the length of the experiment. Results are presented in the same manner as in Fig. 2.

390 Tables

391

392 Table 1: Results from three way fixed factor ANOVAs for $\delta^{15}N$, %N, $\delta^{13}C$, %C for

393 frozen, oven, and desiccator samples of *Ulva* and *Gracilaria* and from two way fixed

394 factor ANOVAs from freshly collected samples.

395

		$\delta^{15}N$			%N			$\delta^{13}C$			%C		
Source	DF	SS	F	P	SS	F	P	SS	F	P	SS	F	P
	Frozen, Oven, and Dessiccator 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		

396
397