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A case against acidifying freshwater macrophytes prior to C and N stable isotope analysis

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Abstract Acidifying organic samples prior to stable isotope analysis is a common practice to eliminate inorganic carbonates; however, it is still unclear what impact this has on $\delta^{13}C$ and $\delta^{15}N$ values. Here we present the first extensive study to test the effects of acidification on freshwater macrophyte carbon and nitrogen isotopic ratios. Focusing on the more productive and ecologically relevant seasons, we collected eleven common macrophyte species (both submerged and emergent), from four lakes in south-central Ontario, Canada. These lakes vary considerably in chemistry, particularly in the inorganic carbon and calcium concentrations. All individual plant samples were equally separated, prepared into acidified and un-acidified treatments, and analyzed for their carbon and nitrogen stable isotope ratios. No significant differences in macrophyte $\delta^{13}C$ or $\delta^{15}N$ were detected between the treatments. Additionally, we found that within-species $\delta^{13}C$ variability was greater in acidified samples. Finally, in assessing the isotopic values of different macrophytes across a chemical gradient at different times throughout the season, it became evident that natural variability exists in both the $\delta^{13}C$ and $\delta^{15}N$ of un-acidified samples within and between species. Based on these results, we assert that pre-analysis acidification of freshwater macrophytes from temperate lakes is unnecessary and not recommended. Additionally, we implore ecologists to acknowledge the macrophyte $\delta^{13}C$ and $\delta^{15}N$ variability in future food web studies.

Keywords Freshwater macrophytes · Sample acidification · Stable isotopes · $\delta^{13}C$ · $\delta^{15}N$

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Introduction

Stable isotope analysis is a popular tool in aquatic ecology. Carbon and nitrogen stable isotopes are particularly useful in food web studies, as $\delta^{15}N$ and the $\delta^{13}C$ values can be used to determine relative trophic position and dietary carbon origin, respectively (DeNiro and Epstein 1981; Rounick and Winterbourn 1986; Peterson and Fry 1987). Stable isotope analysis has been used extensively in terrestrial (e.g., Chamberlain et al. 1997; Ponsard and Arditi 2000), marine (e.g., Fry 1991; Hansson et al. 1997), and freshwater (e.g., Kling et al. 1992; Vander Zanden et al. 1997, 2005) studies. However, despite their wide application in such studies, a fully standardized method for dealing with inorganic carbonates in organic sample

preparation has yet to be developed, making inter-study comparisons challenging. These comparisons are especially tenuous since lake carbonate concentrations are so variable across spatial and temporal scales, and, as such, it is possible that trends could be muted or amplified by methodological differences.

Obtaining accurate isotopic estimations is a fundamental issue in stable isotope ecology. Relative trophic position is determinable based on the assumption that the $\delta^{15}N$ is greater by a predictable amount (on average 3–4 ‰) in a consumer relative to its diet (DeNiro and Epstein 1981; Vander Zanden and Rasmussen 2001; Post 2002); thus, a baseline nitrogen value (reflective of basal food resources) is required to make trophic level estimations. Additionally, since $\delta^{13}C$ enrichment is comparatively minimal ($\sim$1 ‰), it is equally as important to accurately quantify (DeNiro and Epstein 1981). Animal and plant samples often contain carbonates (predominately $CaCO_3$), which are enriched in ^{13}C relative to organismal tissue (Peterson and Fry 1987). Furthermore, the amount of $CaCO_3$ in the tissues of aquatic organisms is in part dependent on lake chemistry (France 1987). In $CaCO_3$-rich environments, aquatic macrophytes can become encrusted as a result of phytoplankton photosynthesis shifting the carbon balance and increasing the pH (Borowitzka 1984). This encrustation is especially prevalent in marl lakes, as they are high in dissolved minerals and typically not highly productive. The various inorganic carbon sources (HCO_3^-, CO_2, CO_3^{2-}) have different isotopic values; however, fractionation during photosynthetic carbon fixation produces organic carbon, which is still depleted in ^{13}C compared to inorganic carbonates that exist in the water body and boundary layer adjacent to aquatic plants (Hecky and Hesslein 1995). To correct for this, samples are often acidified to remove the inorganic carbon (Fry 1984, 1988); however, it remains unclear whether acidification of all samples is a necessary or an advisable step in the preparation process.

Several studies have attempted to assess the effects of acidifying animal samples on carbon and nitrogen isotopic values, but the results are inconsistent. Studies completed on similar organisms have drawn disparate conclusions. For instance, marine invertebrates have been found to have a significant depletion in $\delta^{15}N$ and $\delta^{13}C$ post-acidification (e.g., Jacob et al. 2005), while other studies have found a significant enrichment in $\delta^{15}N$, and no change in $\delta^{13}C$ (e.g., Bunn et al. 1995). Further, studies on marine animals have indicated no change to either $\delta^{15}N$ or $\delta^{13}C$ post-acidification (e.g., Bosely and Wainwright 1999; Serrano et al. 2008). It is unclear which mechanisms are responsible for the changes (or lack thereof) in each of these studies; however, it is predicted that the acidification of samples should result in a depletion of $\delta^{13}C$, since the ^{13}C-enriched carbonates are being lost via ebullition of CO_2. Increases in $\delta^{13}C$ values could also be possible since acid can potentially react with carbon compounds other than carbonates (Serrano et al. 2008), so that organic carboxyl groups are digested instead of or in addition to inorganic carbonates. Similarly, acidification could result in enriched $\delta^{15}N$ values if the acid reacted with nitrile groups, since lighter isotopes have comparatively weaker bonds and faster reaction times than their heavier counterparts (Peterson and Fry 1987). The latter two scenarios provide undesirable outcomes, which if achieved would support the decision to exclude an acidification step from sample preparation.

Determining the $\delta^{13}C$ and $\delta^{15}N$ values of primary producers is especially important for food web studies, since these comprise the baseline of many aquatic ecosystems. More specifically, freshwater macrophytes act as a critical food source for invertebrates in the forms of direct herbivory and detritivory (Newman, 1991; Mendonca et al. 2013). However, very few studies have been completed on the effects of acidifying plant samples, and to our knowledge, none have focused specifically on freshwater macrophytes. One study examined the impact of sample acidification on sea grass (*Enhalus acoroides*), finding no difference in $\delta^{13}C$, and a significant depletion in $\delta^{15}N$ of acidified samples (Bunn et al. 1995). More recently, Brodie et al. (2011) completed a systematic comparison of acidification methods on different terrestrial and aquatic organic matter (excluding macrophytes) and reported that there was significant within- and between-species variability in $\delta^{13}C$. However, since carbon isotopic values in marine, terrestrial, and freshwater environments are markedly different (Peterson and Fry 1987), there remains a need to investigate the effects of acidification on freshwater macrophytes.

Carbon and nitrogen stable isotope ratios are typically determined simultaneously from the same sample; thus, if acidification is required to remove inorganic carbon, it must have a negligible impact on the $\delta^{15}N$ value to make it a viable step in sample

preparation. Furthermore, if there is no impact of acidification on carbon isotopic values of plants from environments rich in $CaCO_3$, then it is unnecessary to acidify samples at all. In this study, we tested the effects of acidification on the $\delta^{13}C$ and $\delta^{15}N$ of common freshwater macrophytes from four different study lakes. We focused on the summer and fall months (samplings in June, July, and October), as macrophytes are highly productive during this time and are therefore most ecologically relevant for higher trophic levels (Mendonca et al. 2013). Furthermore, calcium carbonate is most likely to encrust macrophytes during this time due to increased algal production (Borowitzka 1984). By studying several species of macrophytes from lakes across gradients of inorganic carbon and calcium (to encompass temporal variability), we intend to make an informed recommendation of whether pre-analysis acidification is necessary or advisable as well as quantify the variability in C and N isotopic values among plant species and lakes.

Materials and methods

Study sites

Macrophyte samples were collected from four lakes in south-central Ontario, between 2009 and 2012. Chub Lake is situated in the Muskoka–Haliburton region, located on the Precambrian Shield, whereas Stoney Lake, Buckhorn Lake, and Raven Lake are typical of the Kawartha Lakes region, with limestone catchments. These lakes range in chemistry, particularly the inorganic carbon and calcium concentrations; Chub has very low levels of each of these, followed by Stoney and Buckhorn with intermediate levels and Raven (a marl lake) with high levels (Table 1). These gradients are so pronounced that the macrophytes from Raven showed visual signs of calcification, which was not evident in the other lakes. By sampling lakes across such a large gradient during the summer and fall months, we were able to account for annual changes in calcium carbonate concentration. Other select physical and chemical lake data are listed in Table 1.

Sample collection and analyses

Representative macrophyte samples were collected from the littoral zones of each study lake. In total, 11 common species were collected: northern milfoil (*Myriophyllum sibericum*), common waterweed (*Elodea canadensis*), Richardson's pondweed (*Potamogeton richardsonii*), stiff arrowhead (*Sagittaria rigida*), spiny quillwort (*Isoetes echinospora*), water shield (*Brasenia schreberi*), hard-stem bulrush (*Schoenoplectus acutus*), broad-leaf arrowhead (*Sagittari latifolia*), yellow pond lily (*Nuphar lutea*), fragrant water lily (*Nymphaee odorata*), and sago pondweed (*Potamogeton pectinatus*) (Table 2). The most abundant specimens present at each site were collected; consequently, species and sample numbers are not necessarily the same among lakes.

Water chemistry samples were collected from the littoral zones of each lake at the time of sampling. We measured dissolved organic carbon, dissolved inorganic carbon, calcium, total phosphorous, and total nitrogen concentrations (Table 1) using standard protocols of the Ontario Ministry of the Environment (for methods, see: OME 1983).

Replicate specimens of each macrophyte species were collected when available, stored at 4 °C in transit, and kept frozen in the laboratory until further processing. Thawed samples were rinsed thoroughly with lake and Milli-Q water to ensure the removal of all epiphytes and debris. Individual plants of each species were equally subdivided, so that both acidified and un-acidified treatments were from the same plant, then placed in glass vials, and oven-dried for 24 h at 50 °C. Each dried subsample weighed between 10 and 20 g.

One subsample of each plant was transferred to a pre-combusted (at 450 °C for 4 h) GFF/A filter and treated with a 0.25 mol L^{-1} HCl solution to eliminate $CaCO_3$. Different acidification methods have been used in previous studies; however, we followed the drop-wise method outlined in Jacob et al. (2005). We chose this method because it is more precise than a single douse in acid (Bosely and Wainwright 1999), or an hour-long acid bath (Bunn et al. 1995), and can effectively remove carbonates from samples, unlike the acid fumigation method (Hedges and Stern 1984). Adding the appropriate amount of acid ensured carbonates were eliminated, while minimizing additional chemical effects. As such, between 3 and 10 mL of HCl was added drop-wise to samples until no more CO_2 evolved. The amount of acid added was based on the visual inspection of CO_2 evolution; treatment was stopped shortly after bubbling ceased. The samples

Table 1 Select chemical and physical data for the four temperate study lakes in south-central Ontario

	Chub	Stoney	Buckhorn	Raven
Latitude	45°13′	44°32′	44°29′	44°36′
Longitude	78°59′	78°08′	78°23′	78°54′
Area (ha)	34	2800	3157	107
Max depth (m)	23	32	7	3
pH	7.2	8.7	8.9	8.6
Conductivity (μS cm^{-1})	14.1	189.9	203.7	398.0
Ca (mg L^{-1})	1.5	32.2	34.6	47.9
DIC (mg L^{-1})	0.4	12.2	12.2	25.4
TP (μg L^{-1})	7.6	9.5	14.1	7.9
TN (μg L^{-1})	193.7	383.5	431.4	1048.5
DOC (mg L^{-1})	7.5	5.5	6.4	16.1

from Chub Lake required the smallest volume of acid, whereas the samples from the remaining lakes required notably more. Samples were not rinsed post-acidification to minimize the loss of dissolved organic matter (DOM) (Jacob et al. 2005). The acidified subsamples were transferred from the filters into glass vials and subsequently oven-dried for 24 h at 50 °C. Following this, both acidified and un-acidified samples were ground to uniform powder in their respective vials with a metal spatula, transferred to tin capsules, and weighed prior to stable isotope analyses. Carbon and nitrogen isotopic ratios, as well as percent carbon, were determined using a Micromass Isoprime continuous flow isotope ratio mass spectrometer in the Water Quality Centre at Trent University. International standards were used to calibrate the isotope results. The IAEA certified reference material USGS40 ($\delta^{13}C = -26.4$ ‰) and ($\delta^{15}N = -4.5$ ‰) was used to calibrate the instrument and normalize the results. Casein, a certified protein standard (Elemental Microanalysis, Okehampton), was used as an internal quality control standard. The analytical reproducibility for $\delta^{13}C$ was ±0.04 ‰ and ±0.10 ‰ for $\delta^{15}N$.

Statistical analyses

We tested all of our data for normality using Shapiro–Wilk tests. Since some of the data were not normally distributed, we used nonparametric analyses to determine whether there were any significant differences. For comparing acidified and un-acidified $\delta^{13}C$, $\delta^{15}N$, and %C values, paired-sample Wilcoxon signed-rank tests were used with Bonferroni corrections to identify differences between treatments in the total dataset (across all lakes) and subsets for individual lakes, species, and points in time (group size $n > 5$). To examine whether all sampled data from lakes, species, or sampling dates had any impact on the difference between acidified and un-acidified samples, Kruskal–Wallis tests were applied with Bonferroni corrections to compare average values per treatment and grouping and to determine the critical p values for assessing significance. Kruskal–Wallis tests were also used to assess differences in un-acidified $\delta^{13}C$ and $\delta^{15}N$ values between lake, species, and sampling date. Finally, a Wilcoxon signed-rank test was used on the relative standard deviations (RSD; when $n > 2$) and relative percent deviations (RPD; when $n = 2$) of plants (of the same species and sampling occasion) within each lake to assess whether within-species variability in $\delta^{13}C$ and $\delta^{15}N$ values differed pre- and post-acidification. Statistical analyses were conducted using R statistical language version 3.1.1 (R Core Team 2013) with the MASS package (Venables and Ripley 2002).

Results

We detected no significant differences in macrophyte $\delta^{13}C$ (Wilcoxon signed-rank: $V = 430$, $p = 0.24$; Fig. 1) or $\delta^{15}N$ (Wilcoxon signed-rank: $V = 253$, $p = 0.14$; Fig. 1) between acidified and un-acidified treatments across the four study lakes ($n = 37$). Similarly, no differences emerged between the two treatments within individual lakes for macrophyte

Table 2 Summary statistics of the $\delta^{13}C$ and $\delta^{15}N$ of 11 common freshwater macrophytes from four lakes in south-central Ontario collected between 2009 and 2012; n = sample number; %RSD/RPD are relative standard deviation and relative percent difference, respectively

Species	Date	n	$\delta^{13}C$ (‰)				$\delta^{15}N$ (‰)			
			Acidified		Un-acidified		Acidified		Un-acidified	
			Mean (%RSD/RDP)	Range	Mean (%RSD/RDP)	Range	Mean (%RSD/RDP)	Range	Mean (%RSD/RDP)	Range
Stoney Lake										
Myriophyllum sibericum	07/12	2	−16.4 (8)	−15.8 to −17.1	−16.6 (5)	−17.0 to −16.2	6.1(28)	5.3 to 7.0	6.3 (11)	6.0 to 6.7
Myriophyllum sibericum	10/12	2	−14.7 (3)	−14.9 to −14.4	−14.7 (1)	−14.9 to −14.5	1.0 (32)	0.34 to 1.64	1.3 (90)	0.7 to 2.0
Elodea canadensis	07/12	2	−20.6 (2)	−20.9 to −20.7	−20.8 (1)	−20.9 to −19.9	1.1 (345)	−0.83 to 3.11	1.3 (192)	0.1 to 2.6
Elodea canadensis	10/12	1	−21.3		−21.7		1.2		3.2	
Potamogeton richardsonii	07/12	2	−20.3 (4)	−20.7 to −19.9	−20.7 (5)	−21.2 to −20.1	4.1 (3)	4.0 to 4.1	4.4 (3)	4.4 to 4.5
Buckhorn Lake										
Nuphar lutea	07/12	4	−24.7 (4)	−25.8 to −23.8	−24.7 (3)	−25.5 to −23.9	4.9 (46)	2.6 to 6.9	4.7 (49)	2.2 to 6.8
Sagittaria latifolia	07/12	2	−27.8 (2)	−28.1 to −27.6	−27.8 (3)	−28.2 to −27.4	7.5 (12)	7.0 to 7.9	7.1 (1)	7.0 to 7.1
Potamogeton richardsonii	07/12	3	−19.7 (5)	−20.7 to −18.6	−20.2 (5)	−21.2 to −19.4	4.9 (30)	4.0 to 6.6	5.4 (32)	4.4 to 7.4
Elodea canadensis	07/12	2	−19.5 (6)	−20.1 to −18.9	−19.7 (4)	−20.1 to −19.3	4.4 (32)	3.7 to 5.2	4.5 (31)	3.8 to 5.2
Nymphaee odorata	07/12	2	−27.8 (1)	−27.8 to −27.7	−27.7 (0)	−27.7 to −27.6	6.1 (5)	5.9 to 6.2	5.9 (8)	5.7 to 6.2
Chub Lake										
Isoetes echinospora	06/12	2	−36.0 (4)	−36.7 to −35.3	−35.6 (0)	−35.7 to 35.6	−2.9 (20)	−3.2 to −2.6	−2.3 (7)	−2.3 to −2.2
Isoetes echinospora	07/09	2	−34.0 (9)	−35.5 to −32.4	−32.8 (6)	−33.7 to −31.8	1.6 (96)	0.8 to 2.4	3.0 (24)	2.7 to 3.4
Brasenia schreberi	06/12	2	−24.3 (1)	−24.4 to −24.2	−24.3 (2)	−24.5 to −24.2	−0.4 (91)	−0.6 to −0.2	−0.3 (1)	−0.3
Sagittaria rigida	06/12	2	−30.0 (4)	−30.6 to −29.3	−30.1 (3)	−30.6 to −29.6	1.6 (4)	1.5 to 1.6	1.7 (3)	1.6 to 1.7
Raven Lake										
Schoenoplectus actus	10/12	4	−26.6 (2)	−27.2 to −25.9	−26.8 (3)	−27.5 to −26.0	−4.4 (26)	−5.8 to −3.0	−5.0 (34)	−6.8 to −3.3
Potamogeton pectinatus	10/12	2	−24.8 (12)	−26.3 to −23.2	−24.5 (1)	−24.3 to −24.6	1.5 (100)	0.7 to 2.2	1.3 (19)	1.2 to 1.5
Nymphaee odorata	10/12	1	−24.5		−25.0		−6.6		−6.1	

$\delta^{13}C$ (Stoney: Wilcoxon signed-rank: $V = 34$, $p = 0.20$; Buckhorn: $V = 54$, $p = 0.50$; Chub: $V = 12$, $p = 0.46$; Fig. 2). This was generally also the case for $\delta^{15}N$, although here pairwise comparisons suggested a low degree of differentiation in some cases (Stoney: Wilcoxon signed-rank: $V = 7$, $p = 0.07$; Buckhorn: $V = 43$, $p = 0.86$; Chub: $V = 3$, $p = 0.04$; Fig. 2). Remarkably, no differences were detected in macrophyte $\delta^{13}C$ between treatments in Raven Lake—the marl lake with elevated $CaCO_3$ concentrations (Wilcoxon signed-rank: $V = 18$, $p = 0.32$; Fig. 2). Further, no differences between treatments became apparent within species and sampling time (Wilcoxon signed-rank, all $p >$ Bonferroni-corrected p critical values). Thus, we detected no lake ($\delta^{13}C$: Kruskal–Wallis: $H = 3.24$, $df = 3$, $p = 0.36$; $\delta^{15}N$: Kruskal–Wallis: $H = 3.61$, $df = 3$, $p = 0.31$), species ($\delta^{13}C$: Kruskal–Wallis: $H = 9.64$, $df = 10$, $p = 0.47$; $\delta^{15}N$: Kruskal–Wallis: $H = 10.95$, $df = 10$, $p = 0.36$), or sampling time ($\delta^{13}C$: Kruskal–Wallis: $H = 4.76$, $df = 3$, $p = 0.19$; $\delta^{15}N$: Kruskal–Wallis: $H = 1.73$, $df = 3$, $p = 0.63$) effects related to the impact of acidification. Although no differences were detected between treatments, the $\delta^{13}C$ values of acidified samples were more variable within species than un-acidified samples (Wilcoxon signed-rank: $V = 95$, $p = 0.05$).

Significant differences in un-acidified macrophyte $\delta^{13}C$ were detected among different species (Kruskal–Wallis: $H = 34.21$, $df = 10$, $p < 0.001$; Fig. 2), lakes (Kruskal–Wallis: $H = 20.57$, $df = 3$, $p < 0.001$; Fig. 2), and sampling dates (Kruskal–Wallis: $H = 12.2$, $df = 3$, $p < 0.01$; Fig. 2). Different species from the same lake were found to vary up to 10 ‰, with an overall $\delta^{13}C$ range of 21 ‰ (Fig. 2). In general, pondweed, milfoil, and waterweed samples were found to be relatively more enriched in ^{13}C than other macrophyte samples (Fig. 2). Similar variability was found in un-acidified macrophyte $\delta^{15}N$ (species: Kruskal–Wallis: $H = 22.82$, $df = 10$, $p = 0.01$; lakes: Kruskal–Wallis: $H = 24.47$, $df = 3$, $p < 0.001$; and sampling date: Kruskal–Wallis: $H = 24.20$, $df = 3$, $p < 0.001$; Fig. 3). There was a temporal difference detected in $\delta^{15}N$ of ~5 ‰ in both samples of milfoil taken 3 months apart from Stoney Lake and in quillwort sampled 3 years apart from Chub Lake (Fig. 3). In addition, there was an overall range in $\delta^{15}N$ of 13 ‰ encompassing all species, sampling times, and lakes (Fig. 3).

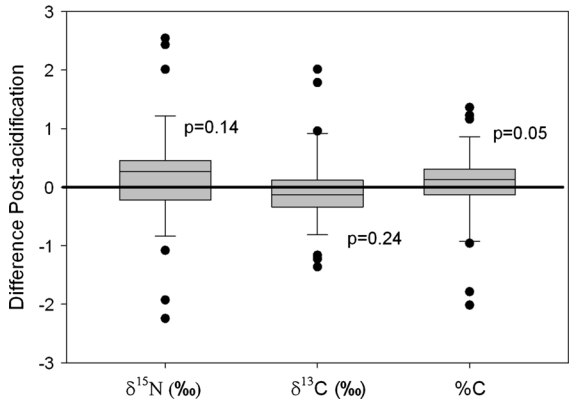

Fig. 1 Paired-sample differences in $\delta^{15}N$, $\delta^{13}C$, and %C measurements of macrophyte samples from acidified and un-acidified treatments. Altogether 37 samples were collected between 2009 and 2012 in four study lakes from south-central Ontario. The *range bars* indicate the maximum and minimum of non-outliers; *boxes* show the interquartile ranges (25–75 %); the *horizontal line* indicates the median; *dots* indicate outliers. Given are *p* values from Wilcoxon signed-rank tests regarding paired-samples differences in the three parameter measurements

Finally, the %C content in acidified samples was found to be greater than that in un-acidified samples across the four study lakes (Wilcoxon signed-rank: $V = 118$, $p = 0.05$; Fig. 1). In more than 70 % of the samples, the percentage of carbon in acidified samples was greater than in their un-acidified counterparts across the four study lakes (Fig. 4). In Chub Lake, however, only half of the samples supported this finding.

Discussion

The unaltered post-acidification $\delta^{13}C$ values suggest that the amount of $CaCO_3$ in these macrophytes was insufficient to impact their carbon isotopic values and that the acidification process did not result in the digestion of organic carboxyl groups in these samples. Carbonates are far more ^{13}C enriched than aquatic macrophyte tissue (Keeley and Sandquist 1992), and, therefore, high $CaCO_3$ content should have resulted in the acidified plant samples being more depleted in ^{13}C. Even in a marl lake, where this effect should be the most pronounced due to elevated $CaCO_3$ levels, no differences in $\delta^{13}C$ values were detected. These results agree with other studies such as Bunn et al. (1995) and Bosely and Wainwright (1999). The unaltered acidified plant $\delta^{13}C$ values from lakes across these

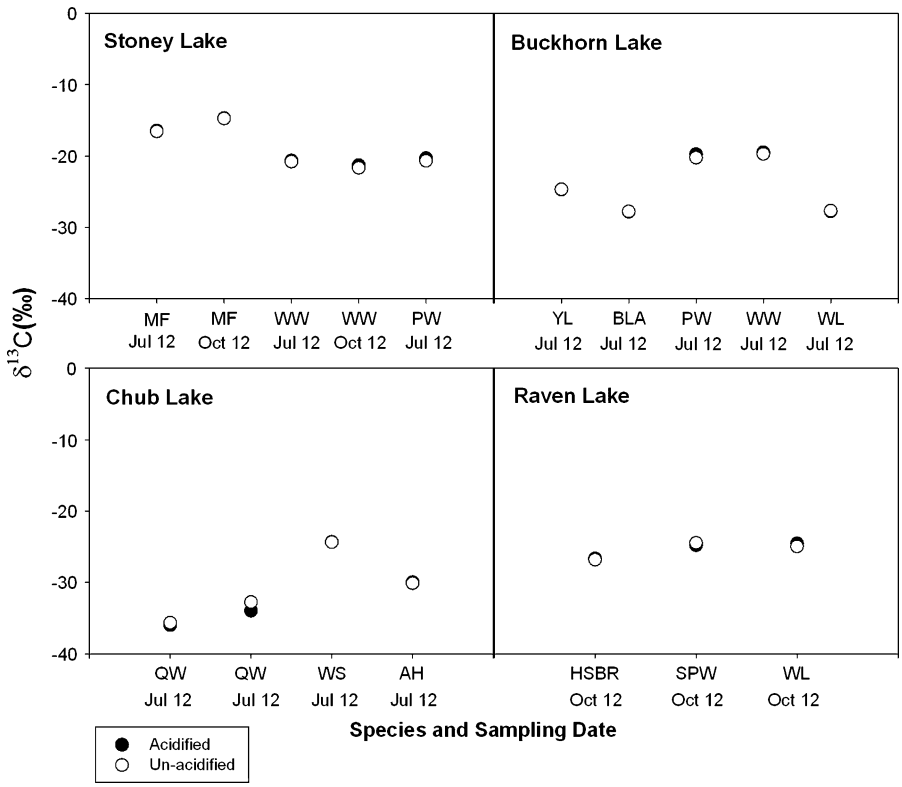

Fig. 2 $\delta^{13}C$ values for the acidified and un-acidified treatments of 11 freshwater macrophytes species collected from 2009 to 2012 from the four study lakes in south-central Ontario. *MF* northern milfoil, *WW* common waterweed, *PW* Richardson's pondweed, *AH* stiff arrowhead, *QW* spiny quillwort, *WS* water shield, *HSBR* hard-stem bulrush, *BLA* broad-leaf arrowhead, *YL* yellow pond lily, *WL* fragrant water lily, and *SPW* sago pondweed

chemical gradients suggest that the concentration of $CaCO_3$ has no bearing on the effects of acidification of macrophytes from temperate freshwater lakes.

Although we did not directly measure the concentration of calcium carbonate in these samples, it is clear that variable concentrations were present. From an observational perspective, the differing length of CO_2 ebullition suggests a presence and a gradient of $CaCO_3$ within the plants. The percent carbon content results provide further support for this, since the majority of acidified samples were found to have greater carbon content than the un-acidified samples. With the precipitation and subsequent loss of the relatively heavy calcium atom from $CaCO_3$ molecules, the remaining organic carbon in the sample becomes proportionally greater, despite the loss of carbon from the $CaCO_3$ molecule. Unsurprisingly, in Chub Lake (the lake with the lowest DIC and Ca concentrations) only half of the samples follow this trend. As such, we postulate that there is a saturation point at which greater $CaCO_3$ concentrations are no longer cumulatively represented in freshwater macrophytes.

Our result of unaltered nitrogen isotopic values post-acidification agrees with those of Bosely and Wainwright (1999), but not Bunn et al. (1995). Soreide et al. (2006) also found that fish, crustacean, and particulate organic matter samples were robust, and the $\delta^{15}N$ values were unaffected by treatments with different acid concentrations. The lack of consistency between our results and those of Bunn et al. (1995) is likely due to a post-acidification rinsing step during their sample processing, which could have led to a loss of dissolved compounds. Brodie et al. (2011) found that rinsing altered isotopic values, %C, and %N, which they attributed to the loss of fine-grained materials, removal of inorganic carbon, and the loss of soluble organic matter. The findings of the Bunn et al. (1995) study are unusual, as the $\delta^{15}N$ values of marine invertebrates were found to be significantly enriched in ^{15}N, while sea grass values were significantly depleted. If any changes in $\delta^{15}N$ were observed, it would be expected that the samples would be ^{15}N enriched or unaltered, since heavier isotopes have stronger bonds (Peterson and Fry 1987), and are

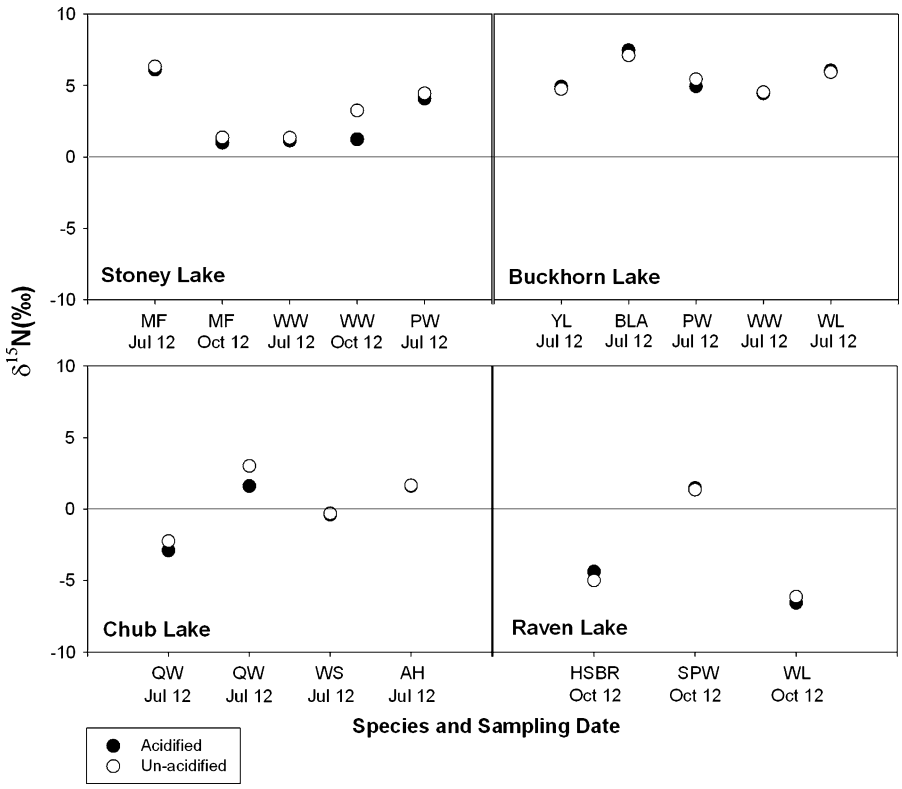

Fig. 3 δ^{15}N values for the acidified and un-acidified treatments of 11 freshwater macrophytes species collected from 2009 to 2012 from the four study lakes in south-central Ontario. *MF* northern milfoil, *WW* common waterweed, *PW* Richardson's pondweed, *AH* stiff arrowhead, *QW* spiny quillwort, *WS* water shield, *HSBR* hard-stem bulrush, *BLA* broad-leaf arrowhead, *YL* yellow pond lily, *WL* fragrant water lily, and *SPW* sago pondweed

thus less likely to break during the acidification process and subsequent chemical reactions.

Acidification also led to increased within-species variability of the macrophyte δ^{13}C values. This observation agrees with the results of Brodie et al. (2011), since they found that there was significant variability in isotopic values, both within and between different acidification methods. In both studies, this increased error is likely a product of some aspect of the acidification process itself; however, the exact mechanism remains unclear at this point. This added variability is problematic, in that it decreases reproducibility and statistical power.

Finally, it is clear that there is species-specific, temporal, and spatial variability in macrophyte δ^{13}C and δ^{15}N values. This has been previously documented; Boon and Bunn (1994) illustrated spatial and temporal differences in both the δ^{15}N and δ^{13}C (>10 ‰) of some plant samples of the same species, while Cloern et al. (2002) showed a seasonal range in estuarine plant δ^{15}N and δ^{13}C of 5–10 ‰. In this study, we have samples of a given species from the same lakes with δ^{15}N values that fluctuate several units per mil over months and years and different species from the same lake that differ drastically in both δ^{13}C and δ^{15}N.

The among-lake variability in macrophyte δ^{13}C is likely a product of water chemistry, specifically DIC. Various studies suggest that differences in source carbon are reflected in the variability in plant δ^{13}C (Osmond et al. 1981; Keeley and Sandquist 1992; Mendonca et al. 2013). In water, inorganic carbon species have different isotopic values; aqueous CO_2 is more depleted in ^{13}C (by ~7–9 ‰) than HCO_3^- and CO_3 (Mook et al. 1974; Hecky and Hesslein. 1995). This disparity in source carbon values is important because unlike terrestrial plants, some aquatic plants have adapted the ability to utilize HCO_3^- in conjunction with CO_2 for photosynthetic processes (Raven et al. 1985; Keeley and Sandquist 1992). CO_2 is the "preferred" chemical species of carbon; however, CO_2 can be limited in aquatic environments, unlike terrestrial ones (Raven et al. 1985). Since δ^{13}C HCO_3^- is more enriched compared to δ^{13}C CO_2 (Mook et al. 1974), plants utilizing bicarbonate could have more enriched δ^{13}C values. This is reflected in the results of

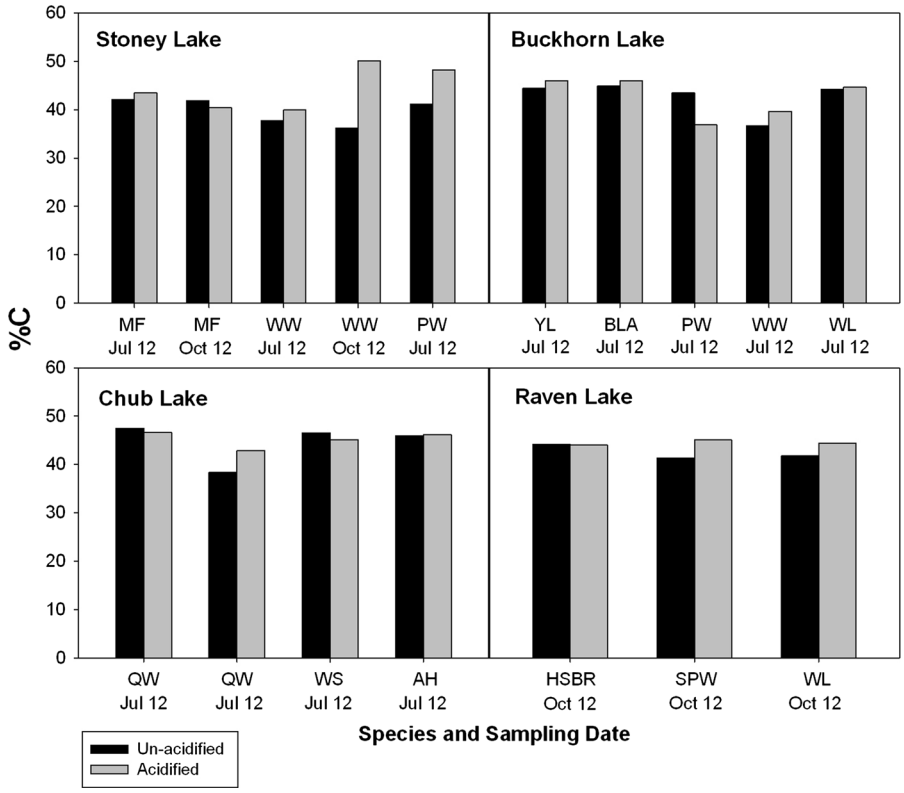

Fig. 4 %Carbon for the acidified and un-acidified treatments of 11 freshwater macrophytes species collected from 2009 to 2012 from the four study lakes in south-central Ontario. *MF* northern milfoil, *WW* common waterweed, *PW* Richardson's pondweed, *AH* stiff arrowhead, *QW* spiny quillwort, *WS* water shield, *HSBR* hard-stem bulrush, *BLA* broad-leaf arrowhead, *YL* yellow pond lily, *WL* fragrant water lily, and *SPW* sago pondweed

our study since milfoils, pondweeds, and waterweeds are all known bicarbonate users (Allen and Spence 1981; Keeley and Sandquist 1995), and samples in these families were found to have relatively more enriched δ^{13}C values. Temporal differences in macrophyte δ^{13}C could also be a product of differences in primary productivity (Gu et al. 1996) and disparate photosynthetic pathways (Maberly and Madsen 2002). Similarly, different sources of nitrogen have distinct δ^{15}N values, which can also change temporally (Peterson and Fry 1987). An example of this is presented in Hodell and Schelski (1998), where they showed a 6 ‰ seasonal difference in sedimenting organic matter due to a shift in dominance from isotopically light phytodetritus (summer) to isotopically heavier organic matter from detrital or heterotrophic sources (winter). Additionally, sources of nitrogen assimilation can differ season to season and species to species (Boon and Bunn 1994).

This variability in isotopic values has implications for food web studies employing stable isotopes as their primary analytical tool. In order to be able to accurately compare food webs from different ecosystems and times, a representative δ^{15}N baseline is required due to the variability of nitrogen sources being incorporated at the base of the food webs. Additionally, δ^{13}C values are often used to infer the dietary carbon sources of aquatic organisms; it is clear from this study that the same species can have different isotopic values in different environments, as well as in the same environment at different times. This underscores the importance of collecting replicate basal resource samples from the site at the time of the study that are ecologically and energetically relevant to the consumers being analyzed.

Our results suggest that the acidification of macrophyte samples from temperate freshwater lakes before stable isotope analysis is not only unnecessary, but also inadvisable. The purpose of acidification is to eliminate any bias that inorganic carbon could have on macrophyte δ^{13}C values, and from this study, it is clear that acidification had no significant impact. Although δ^{15}N differences are more noticeable than δ^{13}C differences between treatments, it is irrelevant when deciding whether or not to acidify, as the impact on δ^{13}C is negligible. Furthermore, the greater δ^{13}C

variability of acidified samples within the same species supports our recommendation against acidification. The added variability could lead to problems with reproducibility and data interpretation. Deciding against acidification will eliminate these issues and is also financially and temporally efficient. Finally, we would like to stress that there can be substantial differences in macrophyte $\delta^{13}C$ and $\delta^{15}N$ values within and among lakes, which is important to acknowledge while establishing a baseline for food web studies. Thus, repeated estimates of baseline values may be necessary even from the same study system.

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