

## Article

# Assigning hydrogen, carbon, and nitrogen isotope values for phytoplankton and terrestrial detritus in aquatic food web studies

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Received 30 September 2013; accepted 22 January 2014; published 29 April 2014

## Abstract

Studies designed to assess the resources supporting aquatic consumers using stable isotope analysis require measurements of the potential end members (basal resources). While some basal resources are easily measured, it is often difficult to physically separate phytoplankton (one potential end member) from other components in seston. Further, terrestrial materials entering aquatic ecosystems undergo diagenetic change, potentially altering isotope composition and making it difficult to assign end member values. We tested techniques for determining the isotopic hydrogen ( $\delta^2\text{H}$ ), carbon ( $\delta^{13}\text{C}$ ), and nitrogen ( $\delta^{15}\text{N}$ ) values of terrestrial and phytoplankton end members in seston. Long term *in situ* leaf decomposition experiments were performed. No appreciable change was found in the isotope values of degraded material (mean change 3.6‰ for  $\delta^2\text{H}$ , 0.0‰ for  $\delta^{13}\text{C}$ , and  $-0.1\%$  for  $\delta^{15}\text{N}$ ). We conclude that the isotope values of terrestrial plant material can be used to assign end members for terrestrial detritus. Using samples collected from 10 lakes with phytoplankton-dominated seston, we compared 3 published methods for estimating the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of phytoplankton. One method, which corrected bulk particulate organic matter (POM) isotope values based on a  $\delta^2\text{H}$  mixing model, accurately predicted measured phytoplankton  $\delta^{13}\text{C}$ . Another method, which used a C:N mixing model to correct bulk POM, also performed well. A new method, proposed here, modified seston isotope values using the difference in C:N of phytoplankton and terrestrial material in a  $\delta^2\text{H}$  mixing model and correctly predicted measured phytoplankton  $\delta^{15}\text{N}$ . We recommend estimating phytoplankton  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  by correcting bulk POM using a  $\delta^2\text{H}$  mixing model, with the C:N modification proposed here for  $\delta^{15}\text{N}$ .

**Key words:** food web, mixing models, phytoplankton, stable isotope analysis, terrestrial organic matter

## Introduction

Stable isotope analysis is a common tool for evaluating resource availability, trophic structure, and consumer basal resource use. In aquatic ecosystems, stable isotope values of carbon ( $^{13}\text{C}/^{12}\text{C}$ :  $\delta^{13}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ :  $\delta^{15}\text{N}$ ), and hydrogen ( $^2\text{H}/^1\text{H}$ :  $\delta^2\text{H}$ ) can be employed to quantify the use of terrestrial (allochthonous) organic matter by aquatic consumers (Marcarelli et al. 2011). Methodological difficulties arise when quantifying consumer basal resources using mixing models, however, because assigning appropriate end member values for some source materials is complicated. For example, phytoplankton are

often difficult to physically separate from bulk seston (Hamilton et al. 2005). In addition, some materials with long residence times (e.g., pelagic and benthic detritus) may undergo changes in isotope ratios over time. Although H isotopes may present an advantage over C and N isotopes owing to the large end member separation (Doucett et al. 2007), uncertainties remain in estimating the incorporation of environmental water in consumer tissues (Solomon et al. 2009), diagenetic changes of end member isotope values (Macko et al. 1983), and the photosynthetic discrimination relative to water that results in the depletion of the H isotope deuterium in phytoplankton.

Live or recently-shed tree leaves are often used to estimate the isotope value of terrestrial end members (Jones et al. 1998, Caraco et al. 2010, Babler et al. 2011, Cole et al. 2011, Francis et al. 2011, Solomon et al. 2011, Cole and Solomon 2012, Wilkinson et al. 2013). In some cases, the isotope values of live leaves are indistinguishable from the isotope values of the often terrestrially derived surface dissolved organic matter (DOM; Wilkinson et al. 2013); however, whether the isotope values of leaves undergoing decomposition change appreciably over time is unclear, potentially confounding the use of live leaves as the end member (Fernandez et al. 2003).

Previous diagenesis experiments suggest that while the  $\delta^{13}\text{C}$  of leaves may not change appreciably, the change in  $\delta^{15}\text{N}$  can be substantial (Caraco et al. 1998). For H isotopes, microbial degradation of plant material could result in some incorporation of environmental water, causing an increase in the detrital terrestrial  $\delta^2\text{H}$  value because water generally has much more positive  $\delta^2\text{H}$  values than leaves (Doucett et al. 2007, Solomon et al. 2009, Soto et al. 2013). The extent to which environmental water incorporation affects the isotope value of decomposing leaves in aquatic habitats is unknown. Differential decomposition of detrital components such as lipids and structural compounds (e.g., lignin) may cause shifts in isotope composition (Fernandez et al. 2003). Large and significant shifts during decomposition would lead to incorrect representation of the terrestrial  $\delta^2\text{H}$  end member when using live leaves.

Directly measuring and assessing the isotope value of the phytoplankton end member is difficult because particulate organic matter (POM) in the seston is typically a mixture of autochthonous and allochthonous material (Marty and Planas 2008, Gu et al. 2011). One method for estimating the phytoplankton end member isotope value is from the average photosynthetic discrimination between phytoplankton and the inorganic substrate utilized in biosynthesis. For example,  $\delta^{13}\text{C}$  values can be calculated based on the discrimination ( $\epsilon_c$ ) between phytoplankton and the dissolved inorganic carbon (DIC) in the water. Hydrogen values can be calculated based on the discrimination ( $\epsilon_H$ ) between phytoplankton and water. In these cases,  $\epsilon_c$  and  $\epsilon_H$  need to be known either from cultures (Caraco et al. 2010, Solomon et al. 2011) or from isolated samples of phytoplankton (Vuorio et al. 2006, Caraco et al. 2010). Estimates of average  $\epsilon_c$  for groups of systems can also be generated using the linear regression method of Mohamed and Taylor (2009; see methods below). Discrimination values are variable within and among systems (Laws et al. 1995, Bade et al. 2006), however, and often

necessitate alternative approaches for estimating phytoplankton isotope values.

Marty and Planas (2008) evaluated 5 methods for determining phytoplankton  $\delta^{13}\text{C}$  in freshwater systems and concluded that estimating values based on an assumed discrimination factor is the least accurate. They also evaluated the use of POM and primary consumer  $\delta^{13}\text{C}$  values as substitutes for phytoplankton  $\delta^{13}\text{C}$ . As the authors note, however, these methods would not be suitable for food web studies because an implicit assumption of such investigations is that both POM and consumers are a mixture of resources, not simply composed of phytoplankton. Alternatively, they recommended correcting the  $\delta^{13}\text{C}$  of POM for phytoplankton biomass based on the carbon to chlorophyll *a* ratio (C:Chl-*a*) or using isolated phytoplankton samples to determine system-specific phytoplankton isotope values.

Alternative methods for correcting bulk POM  $\delta^{13}\text{C}$  based on the phytoplankton proportion of the POM pool have recently been published. Cole et al. (2011) utilized the large differences between phytoplankton and terrestrial  $\delta^2\text{H}$  values to calculate the terrestrial fraction of POM ( $\phi_T$ ) using a linear mixing model (see Table 1). The phytoplankton fraction ( $1 - \phi_T$ ) of POM was used to calculate the  $\delta^{13}\text{C}$  of phytoplankton ( $\delta^{13}\text{C}_A$ ) based on the  $\delta^{13}\text{C}$  of POM. Francis et al. (2011) used a similar method of C:N ratios of phytoplankton and terrestrial material to derive  $\phi_T$ . Both methods can also be used to derive the  $\delta^{15}\text{N}$  of phytoplankton by substituting  $\delta^{15}\text{N}_{\text{POM}}$  and  $\delta^{15}\text{N}_T$ . Both methods are theoretically unsuitable for systems with a highly allochthonous (>70%) POM pool, however, because the calculation includes a denominator bound between 0 and 1 (Table 1).

We evaluated methods and assumptions employed for determining phytoplankton and terrestrial organic matter end members for aquatic food web studies. Using the measured isotope values of isolated phytoplankton samples and corresponding POM samples in 10 lakes, we assessed the accuracy of the recent phytoplankton  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  estimation methods presented in the literature. We also examined the variability in  $\delta^2\text{H}$  of phytoplankton and calculated  $\epsilon_H$  for the 10 lakes because there are few published values of  $\epsilon_H$  for lakes. Additionally, we performed *in situ* decomposition experiments and sampled terrestrial material during degradation in both lotic and lentic systems to determine if there is a diagenetic impact on the isotope values of terrestrial leaves. From our analyses, we recommend methods for estimating end members for food web mixing model studies.

**Table 1.** Methods used to estimate phytoplankton  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . The equations and explanation of how variables in the equations are obtained are included. Output is the isotope species of the phytoplankton end member being estimated by the method.  $\delta X$  is used in equations where  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  values are used to calculate the respective phytoplankton end member. The parameter value for  $\rho$  was derived as described in the text.

Method	Citation	Output	Equation 1	Equation 2	Variables
A	Cole et al. (2011)	$\delta^{13}\text{C}_A$ , $\delta^{15}\text{N}_A$	$\phi_T = \frac{(\delta^2\text{H}_{\text{POM}} - \delta^2\text{H}_A)}{(\delta^2\text{H}_T - \delta^2\text{H}_A)}$	$\delta X_A = \frac{\delta X_{\text{POM}} - (\phi_T \times \delta X_T)}{(1 - \phi_T)}$	$\delta^2\text{H}_T$ = live leaves $\delta^2\text{H}_A$ = equation 1 $\delta^2\text{H}_{\text{POM}}$ = sampled
B	Francis et al. (2011)	$\delta^{13}\text{C}_A$ , $\delta^{15}\text{N}_A$	$\phi_T = \frac{(C:N_{\text{POM}} - C:N_A)}{(C:N_T - C:N_A)}$	$\delta X_A = \frac{\delta X_{\text{POM}} - (\phi_T \times \delta X_T)}{(1 - \phi_T)}$	$C:N_T$ = live leaves $C:N_A = 6.8^*$ $C:N_{\text{POM}}$ = sampled
C	Mohamed and Taylor (2009)	$\delta^{13}\text{C}_A$	$\varepsilon_C = \frac{\delta^{13}\text{C}_T - b}{m}; \text{ where}$ $\delta^{13}\text{C}_{\text{POM}} = \delta^{13}\text{C}_T \times m - b$	$\delta^{13}\text{C}_A = \delta^{13}\text{C}_T - \varepsilon_C$	$\delta^{13}\text{C}_T$ = sampled b = intercept m = slope $\delta^{13}\text{C}_T$ = sampled
D	This study	$\delta^{15}\text{N}_A$	$\phi_T = \frac{(\delta^2\text{H}_{\text{POM}} - \delta^2\text{H}_A)}{(\delta^2\text{H}_T - \delta^2\text{H}_A)}$	$\delta^{15}\text{N}_A = \frac{\delta^{15}\text{N}_{\text{POM}} - (\phi_T \times \rho \times \delta^{15}\text{N}_T)}{(1 - \phi_T \times \rho)}$	$\delta^2\text{H}_T$ = live leaves $\delta^{15}\text{N}_T$ = live leaves $\delta^2\text{H}_A$ = equation 1 $\delta^2\text{H}_{\text{POM}}$ = sampled $\rho = 0.3$

## Methods

### Study sites

The terrestrial decomposition incubations occurred in Peter Lake (89°32'W; 46°13'N) in northern Michigan (USA) and Steger Creek (78°16'W; 37°56'N) in central Virginia (USA). Peter Lake is a small (2.4 ha), colored lake, and Steger Creek is a second-order stream. Both systems have forested watersheds. The isolated phytoplankton samples were from 10 lakes located in the Northern Highlands Lake District (USA; Wilkinson et al. 2013).

### Terrestrial end member

Leaf decomposition experiments were conducted in Peter Lake and Steger Creek to investigate changes in leaf  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  during decomposition. Fresh leaves were collected from sugar maple (*Acer saccharum*) growing near the Peter Lake shoreline and American hornbeam (*Carpinus caroliniana*) in forests adjacent to Steger Creek. Mesh bags deployed directly above the bottom sediments and fully submerged underwater were filled with 25 leaf disks 1-inch in diameter. Replicate (n = 3) leaf packs were collected on 5 dates in 2011 from both Peter Lake (Jun–Aug) and Steger Creek (Sep–Nov) at intervals of 0, 1, 2, 4, and 8 weeks. Three additional leaf packs were left to incubate in Peter Lake for a year and were collected in May 2012. Water was sampled on the first and last sampling dates at each site, filtered (Whatman GF/F filters), and stored in air-tight glass vials at 4 °C for  $\delta^2\text{H}_2\text{O}$

analysis. Particulate samples from the mesh bags were manually cleaned, oven-dried at 60 °C, and ground into fine powder.

Additionally, samples of decomposing leaves and benthic samples of terrestrial fragments >2 mm were collected from Peter Lake for  $\delta^2\text{H}$  analysis. The  $\delta^2\text{H}$  values of the benthic samples and terrestrial fragments were compared with isotope values from the controlled decomposition experiments. To test whether trees in the Peter Lake watershed have varying  $\delta^2\text{H}$  values due to different water sources within the watershed, we sampled sugar maple leaves along a 30 m transect away from the lake shore into the forest.

One-way ANOVAs ( $\alpha = 0.05$ ) were used to examine the significance of changes in the leaf pack  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  values during the decomposition experiments. If significant differences were found, multiple comparisons (Tukey-Kramer test) were used to determine where the differences occurred among treatments.

### Phytoplankton end member

Bulk phytoplankton and POM isotope samples were collected in 10 lakes as part of a larger isotope survey (Wilkinson et al. 2013). POM samples were taken at a depth of 0.5 m in each lake, vacuum filtered onto 40 mm MicronSep Cellulosic filters (nominal pore size = 0.8  $\mu\text{M}$ ), back-rinsed, and dried at 60 °C. We obtained phytoplankton samples by net tows (80  $\mu\text{m}$  mesh size) in the epilimnion of these lakes. These samples were inspected under a dissecting microscope to confirm that

the samples consisted mostly of identifiable algae; visible nonphytoplankton particles were removed. The cleaned phytoplankton samples were prepared and analyzed in the same manner as the POM samples. Net phytoplankton and POM samples were analyzed for C, N, and H isotopes. Filtered water samples were collected from each lake and preserved with sodium azide for  $\delta^{13}\text{C}$ -DIC analysis. The equilibrium value of  $\delta^{13}\text{CO}_2$  was calculated using  $\delta^{13}\text{C}$ -DIC value, pH, and temperature (Zhang et al. 1995). Water for  $\delta^2\text{H}_2\text{O}$  analysis from each lake was collected and preserved as described above.

### Isotope analysis

$\delta^2\text{H}$  isotope samples were analyzed at the Colorado Plateau Stable Isotope Laboratory (CPSIL). The isotope value of nonexchangeable H was measured following the methods of Doucett et al. (2007). A bench-top equilibration procedure was used to correct for the exchange of H between ambient water vapor and a set of standards including keratin; caribou and cow hoof; kudo horn; moose, bear and elk hair; baleen; feathers; and chitin; as well as *Cladophora* sp. (an alga).

H-exchange of the *Cladophora* standard is likely similar to phytoplankton; however, differential exchange introduces error in our analysis in 2 ways. First, the assignment of the H isotope value of the terrestrial end member could be incorrect. For example, if H-exchange were as high as 20%, the measured value would be  $-126\text{‰}$  for a hypothetical leaf with an actual value of  $-135\text{‰}$  under typical lab conditions. Similarly, if phytoplankton H-exchange were as high as 20%, photosynthetic isotope discrimination would be underestimated by 15%. Measured H-exchange for many materials is  $<20\text{‰}$  (Chesson et al. 2009), however, and differential H-exchange relative to a standard is likely even lower. These considerations suggest an uncertainty caused by H-exchange of  $\pm 5\text{‰}$  or less for most of the H isotope values considered here.

The analytical precision for dried organic matter replicate samples at CPSIL is 2‰ for  $\delta^2\text{H}$  (M. Caron, CPSIL, Mar 2013, pers. comm.).  $\delta^2\text{H}$  in water samples was analyzed using cavity ring-down laser spectroscopy;  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotope samples were analyzed at the University of Virginia. Typical reproducibility for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  based on replicate analyses is  $>0.3\text{‰}$ . Steger Creek  $\delta^{13}\text{C}$  data were not included in the analysis presented here because an instrument problem produced C yields too low for accurate analysis. All isotope values are expressed in per mil (‰) notation relative to the standards: Vienna Standard Mean Ocean Water (V-SMOW) for  $\delta^2\text{H}$ , Pee Dee Belemnite (PDB) for  $\delta^{13}\text{C}$ , and atmospheric  $\text{N}_2$  for  $\delta^{15}\text{N}$ .

### Phytoplankton discrimination factors

Isotopic differences between a photosynthetic organism and the photosynthetic substrate can be expressed several ways. We used the discrimination factor,  $\epsilon_{\text{H}}$ , determined for each of the 10 lakes using the following equation:

$$\epsilon_{\text{H}} = \frac{\delta^2\text{H}_{\text{A}} - \delta^2\text{H}_{\text{O}}}{1 + \left(\frac{\delta^2\text{H}_{\text{O}}}{1000}\right)}, \quad (1)$$

where  $\delta^2\text{H}_{\text{O}}$  and  $\delta^2\text{H}_{\text{A}}$  is the H isotope value of the lake water and isolated phytoplankton sample, respectively. Equation 1 was also used to calculate the analogous C discrimination factor,  $\epsilon_{\text{C}}$ , by using  $\delta^{13}\text{CO}_2$  in each lake instead of  $\delta^2\text{H}_2\text{O}$ , and  $\delta^{13}\text{C}_{\text{A}}$  instead of  $\delta^2\text{H}_{\text{A}}$  from the 10 lakes. A linear regression analysis was performed to determine if  $\epsilon_{\text{H}}$  or  $\epsilon_{\text{C}}$  for the 10 lake samples was correlated with commonly measured variables. Candidate variables included Chl-*a*, dissolved organic C, DIC, pH, total phosphorus (TP), total N, and water color (absorbance at 440 nm). For a detailed description of sample methods, see Wilkinson et al. (2013).

### Evaluation of phytoplankton $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ estimation methods

Three methods (Table 1) from the literature that were not evaluated in Marty and Planas (2008) were applied to the POM isotope dataset to evaluate each method's ability to recover the measured values of  $\delta^{13}\text{C}_{\text{A}}$  (Table 2). Method A from Cole et al. (2011) corrects the bulk POM  $\delta^{13}\text{C}$  value based on the terrestrial fraction of the POM calculated from a  $\delta^2\text{H}$ -based mixing model. Method B from Francis et al. (2011) is identical to Method A except that the mixing model uses C:N values. Methods A and B were also used to derive an estimate of phytoplankton  $\delta^{15}\text{N}$ , which was compared to  $\delta^{15}\text{N}_{\text{A}}$  ( $\delta^{15}\text{N}$  of the isolated phytoplankton samples). Both methods, as published, used samples of live leaves as the terrestrial end member. Method C uses an average estimate of  $\epsilon_{\text{C}}$  derived from a population of lakes in equation 1. The terrestrial end member isotope values ( $\delta^2\text{H}_{\text{T}}$ ,  $\delta^{13}\text{C}_{\text{T}}$ , and  $\delta^{15}\text{N}_{\text{T}}$ ) were from 81 leaf samples from Northern Highlands Lake District (Solomon et al. 2011). The terrestrial C:N value ( $\text{C:N}_{\text{T}} = 26.8$ ) was estimated from 22 leaf samples from the same study. The value of  $\text{C:N}_{\text{A}}$  was the average of freshwater phytoplankton C:N values presented in Vuorio et al. (2006).

A new method (D) for estimating phytoplankton  $\delta^{15}\text{N}$  was also proposed and evaluated. The variation in the ratio of H:C in most biological materials is small (Anderson 1995); thus, the same fraction of seston that is algal or terrestrial (based on  $\delta^2\text{H}$ , Method A) can provide a direct estimate of  $\delta^{13}\text{C}_{\text{A}}$ . Because the molecular C:N of



**Table 2.** Measured values of isotopes of C, N, and H in phytoplankton and in seston, along with the bulk C:N ratio by atoms in the seston of 10 lakes in northern Wisconsin and Michigan (USA). All isotope values are expressed as per mil (‰) relative to the appropriate standards (see text).

Lake	Phyto $\delta^2\text{H}$	Phyto $\delta^{13}\text{C}$	Phyto $\delta^{15}\text{N}$	POM $\delta^2\text{H}$	POM $\delta^{13}\text{C}$	POM $\delta^{15}\text{N}$	POM C:N	$\delta^2\text{H}_2\text{O}$	$\delta^{13}\text{CO}_2$
Allequash	-226.8	-31.9	0.0	-194.4	-28.5	1.6	8.30	-66.4	-17.5
Big Lake	-209.3	-31.2	5.4	-206.8	-29.9	3.7	8.88	-60.3	-13.4
Big Muskellunge	-167.9	-19.8	4.1	-172.4	-22.3	2.4	12.58	-43.2	-10.9
Deadwood	-180.9	-33.9	1.2	-134.9	-29.3	0.7	10.63	-45.9	-17.5
Found	-236.7	-26.7	3.6	-175.9	-25.0	3.3	12.96	-49.7	-14.5
Inkpot	-200.9	-34.9	1.8	-171.0	-32.7	1.4	6.23	-62.1	-17.5
Little Arbor Vitae	-227.0	-24.0	3.4	-244.9	-25.7	-0.2	10.97	-53.9	-10.7
Presque Isle	-202.4	-28.9	4.8	-191.8	-27.1	3.9	8.77	-53.8	-13.5
Sparkling	-193.2	-26.5	4.2	-172.3	-26.6	0.7	10.31	-48.2	-12.1
Tenderfoot	-222.7	-29.5	3.5	-186.9	-30.0	2.3	8.36	-64.4	-14.6

algae is lower than that of terrestrial organic matter, however, more of the molecular N in seston is likely from the algal fraction. For example, if  $\phi_T$  is 0.5, the C:N ratio of phytoplankton is twice as high as  $1 - \phi_T$ . In Method D, to account for the inconsistency in C:N between the end members, a correction factor  $\rho$  was used to modify Method A. After evaluating all potential values of  $\rho$ , 0.3 was selected because it yielded the best fit to the measured data, suggesting that the C:N of terrestrial inputs is 3 times higher than that of phytoplankton.

The calculated  $\delta^{13}\text{C}_A$  from Methods A–C and calculated  $\delta^{15}\text{N}_A$  from Methods A, B, and D were compared to the directly measured  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of the phytoplankton sampled in the corresponding lake to quantify how well the methods recovered the phytoplankton isotope values. To compare methods, the absolute deviation of the estimated value from the measured value was calculated and averaged across all lakes for each method. Linear regression analysis was used to determine if the slope and intercept of measured versus estimated phytoplankton isotope values was significantly different from unity.

## Results

### Terrestrial decomposition experiments

Significant mass loss over time was observed in both Peter Lake ( $p < 0.01$ ) and Steger Creek ( $p < 0.01$ ). By week 8, only 11% of the original leaf mass remained in Peter Lake while 49% remained in Steger Creek. Overall, there was no correlation between  $\delta^2\text{H}$  and percent mass loss. The average water  $\delta^2\text{H}$  values in Peter Lake and Steger Creek were  $-45.1$  and  $-36.4$ ‰, respectively.

In both systems, there was a decline in leaf  $\delta^2\text{H}$  ( $-8.0$

and  $-8.7$ ‰ in Peter Lake and Steger Creek, respectively) during the first week of incubation, followed by more positive isotope values over the next 7 weeks (Fig. 1). For Peter Lake, there was no significant difference (using one-way ANOVA for analyses) between leaf  $\delta^2\text{H}$  values among weeks (Fig. 1a;  $F_{4,10} = 2.15$ ,  $p = 0.15$ ). The average change in leaf  $\delta^2\text{H}$  value after 8 weeks was  $-0.5$ ‰. The average change in  $\delta^2\text{H}$  of the leaf packs that were incubated for a year in Peter Lake was  $-10.0$ ‰, which was significantly less than weeks 0 and 8 ( $F_{5,12} = 3.9$ ,  $p = 0.02$ ). The largest average change in  $\delta^{13}\text{C}$  for Peter Lake was  $-0.3$  and  $-0.1$ ‰, respectively (Fig. 1b). The changes in C isotope values during the incubation determined were insignificant ( $F_{4,9} = 0.71$ ,  $p = 0.60$ ), as were the changes in N isotope values ( $F_{4,10} = 0.36$ ,  $p = 0.83$ ). In contrast, Steger Creek leaf  $\delta^2\text{H}$  values over the 2 months were significantly different (Fig. 1c;  $F_{4,10} = 13.30$ ,  $p < 0.001$ ). The change in  $\delta^2\text{H}$  between weeks 0 and 8 was  $+1.8$ ‰. The average change in  $\delta^{15}\text{N}$  for Steger Creek was  $-0.1$ ‰; however, none of the changes in  $\delta^{15}\text{N}$  values were significantly different between weeks (Fig. 1d;  $F_{4,10} = 1.3$ ,  $p\text{-value} = 0.33$ ).

### Terrestrial transect sampling

The  $\delta^2\text{H}$  values of the sugar maple leaf transect in the Peter Lake watershed ranged from  $-135.4$  to  $-120.6$ ‰. There was no correlation between leaf  $\delta^2\text{H}$  and distance from shore. The mean  $\delta^2\text{H}$  value of benthic terrestrial fragments from Peter Lake was  $-144.1$ ‰ (SD 14.5), ranging from  $-124.1$  to  $-159.0$ ‰. There were no correlations with depth or distance from shore and no significant difference in  $\delta^2\text{H}$  between the leaf transect samples and the benthic terrestrial fragment samples (one-way ANOVA;  $F_{1,10} = 1.3$ ,  $p\text{-value} = 0.21$ ).

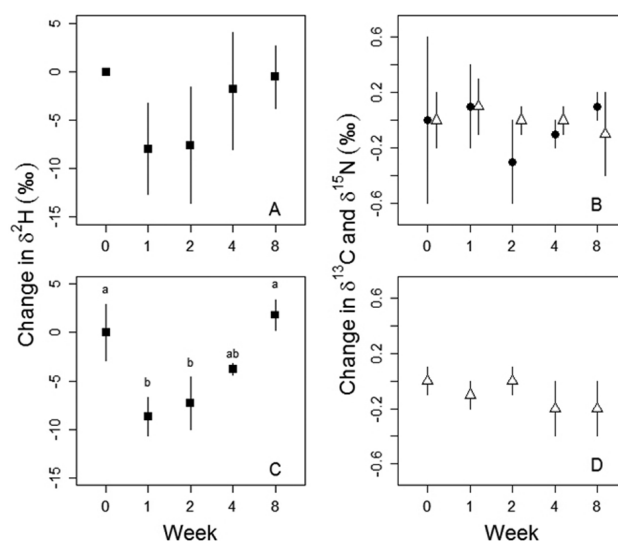
### Phytoplankton discrimination values: $\epsilon_H$ and $\epsilon_C$

Mean  $\epsilon_H$  calculated using equation 1 for the 10 lakes was  $-160.9$  (SD 19.8), ranging from  $-130.3$  to  $-182.9$ ‰.  $\epsilon_H$  was correlated to TP concentration ( $y = 20.4x + 155.5$ ;  $R^2 = 0.51$ ;  $p$ -value = 0.02). Additionally,  $\epsilon_H$  was related to the Chl-*a* concentration ( $y = 0.32x - 39.8$ ;  $R^2 = 0.40$ ;  $p$ -value = 0.05). The phytoplankton  $\delta^{13}C$  in the net samples from the 10 lakes ranged from  $-34.9$  to  $-19.8$ ‰, and the phytoplankton  $\delta^{15}N$  ranged from 0.0 to 5.4‰ (Table 2). The mean  $\epsilon_C$  was  $-14.7$ ‰ (SD 2.7), ranging

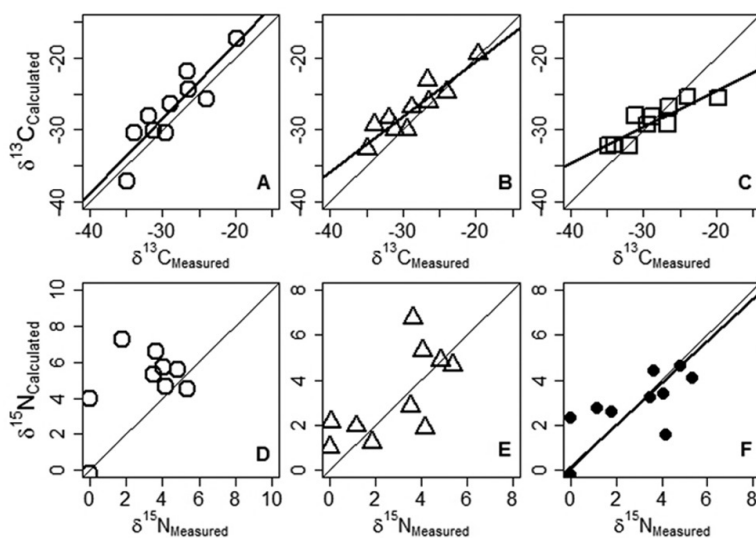
from  $-8.9$  to  $-17.6$ ‰.  $\delta^{13}C_A$  and  $\epsilon_C$  were not significantly correlated with any other variables.

### Phytoplankton $\delta^{13}C$ and $\delta^{15}N$ estimation methods

Method A was the only method that resulted in estimated  $\delta^{13}C_A$  not significantly different from measured values (Table 3). When measured and estimated values were regressed, the slope and intercept were not significantly different from 1 and 0, respectively (Fig. 2a). Method B produced estimates of  $\delta^{13}C_A$  that when compared to the



**Fig. 1.** Average change in isotope value of leaf packs during incubation for (A) Peter Lake:  $\delta^2H$ ; (B) Peter Lake:  $\delta^{13}C$  (circles) and  $\delta^{15}N$  (triangles); (C) Steger Creek:  $\delta^2H$  (similar letters above data point indicate no significant difference between weeks); and (D) Steger Creek:  $\delta^{15}N$ . There are no  $\delta^{13}C$  data for Steger Creek due to low yields (see Methods). Error bars indicate the standard deviations of the replicate samples.



**Fig. 2.** Measured vs. calculated  $\delta^{13}C$  and  $\delta^{15}N$  values using 4 methods (described in Table 1). The thin line is the 1:1 line and the bold line is the linear regression when significant. (A) Method A for  $\delta^{13}C_A$ ; (B) Method B for  $\delta^{13}C_A$ ; (C) Method C for  $\delta^{13}C_A$ ; (D) Method A for  $\delta^{15}N_A$ , not all points are shown due to scaling of the y-axis; (E) Method B for  $\delta^{15}N_A$ ; (F) Method D for  $\delta^{15}N_A$ .

**Table 3.** Results of linear regression analyses (Fig. 2). Slope Unity indicates whether the slope of the regression is not significantly different from 1, and Intercept Zero indicates whether the intercept is not significantly different from 0. Best fitting methods would have an answer of “yes” for both Slope Unity and Intercept Zero. Method letters correspond with those presented in Table 1.

Isotope	Method	Mean Absolute Deviation	Slope	Intercept	R <sup>2</sup>	p-value	Slope Unity	Intercept Zero
$\delta^{13}\text{C}$	A	2.5	1.04	2.79	0.8	>0.01	Yes	Yes
$\delta^{13}\text{C}$	B	1.9	0.77	-5.09	0.8	>0.01	Yes	No
$\delta^{13}\text{C}$	C	1.9	0.5	-14.58	0.8	>0.01	No	No
$\delta^{15}\text{N}$	A	10.1	-3.3	17.1	0.1	0.23	No	No
$\delta^{15}\text{N}$	B	1.4	-0.3	1.3	0.5	0.11	No	No
$\delta^{15}\text{N}$	D	1.4	0.93	0.17	0.5	0.02	Yes	Yes

measured values had a slope at unity, but the intercept was significantly different from 0 (Fig. 2b). Method C produced estimates of  $\delta^{13}\text{C}_A$  that when compared to the measured values differed from unity in both slope and intercept (Fig 2c). Both Methods A and B produced estimates of  $\delta^{15}\text{N}_A$  with regression slopes significantly different from unity (Table 3; Fig. 2d and e). Method D was the only method that produced estimates of  $\delta^{15}\text{N}_A$  with regression slopes not significantly different from unity (Fig. 2f).

## Discussion

### Decomposition of terrestrial detritus

The incubated terrestrial leaf samples had insignificant changes in stable isotope values of H, C, and N between the initial and final weeks of incubation. An initial depletion of leaf deuterium within the first week of decomposition, followed by a gradual enrichment over the next 7 weeks, was observed in both Peter Lake and Steger Creek. The initial depletion observed in both systems was smaller than the variability usually reported among species pooled for the terrestrial end member (Cole et al. 2011, Solomon et al. 2011). Because different leaf compounds have different  $\delta^2\text{H}$  values and are leached and degraded at varying rates, changes would be expected in the bulk level of the  $\delta^2\text{H}$  signature of leaves decomposing over time (DeBond et al. 2013). The initial depletion may be due to leaching of soluble compounds and exchange of H between leaves and water, while mass loss thereafter was likely due to biotic decay of the leaves (Ostrowsky 1997).

Although the pattern of initial rapid decline in leaf  $\delta^2\text{H}$  followed by a gradual increase was observed in both systems, the statistical significance of the differences between leaf  $\delta^2\text{H}$  varied between the 2 systems. In Peter Lake, there were no significant differences among leaf  $\delta^2\text{H}$  values from different sampling times. With only 11% of the leaf mass remaining after 8 weeks, the leaf  $\delta^2\text{H}$  was

nearly equal to the initial values. While the leaf packs that incubated in Peter Lake for a year had slightly lower  $\delta^2\text{H}$  values than those from weeks 0–8 of the decomposition experiment, there was not a substantial difference in leaf  $\delta^2\text{H}$ .

Even if the largest difference in leaf  $\delta^2\text{H}$  values between weeks (-8.7‰ in Steger Creek) was applied as a correction for the entire pool of terrestrial resources in an aquatic system, the remaining difference between the aquatic and terrestrial  $\delta^2\text{H}$  values is still large enough that the impact of the diagenetic shift would be minimal in a mixing model. Applying the average change in leaf  $\delta^2\text{H}$  to the terrestrial end member has an even smaller impact on the mixing model outcome. The consistency of the leaf  $\delta^2\text{H}$  in the decomposition experiment was similar to observations of no differences in live leaves along the transect away from the shoreline and no differences in benthic leaf fragments that had been decomposing for an unknown amount of time in Peter Lake.

For Steger Creek, there were significant differences between specific leaf  $\delta^2\text{H}$  values over time, reflecting the rapid decline of leaf  $\delta^2\text{H}$  during the first week (Fig. 1c). The decrease of leaf  $\delta^2\text{H}$  during the first week could be due to the leaching of more deuterium-rich plant compounds and mechanical breakdown by moving water. Additionally, water temperatures during the Peter Lake incubation were more stable than the Steger Creek experiment, which occurred in late autumn as temperatures were declining. Rapid temperature changes in the shallow stream could have contributed to the small, yet significant, differences in isotope composition observed in Steger Creek but not Peter Lake (Andrews et al. 2000).

### Phytoplankton isotope values

The average of the 10 values of  $\epsilon_{\text{H}}$  calculated using isolated phytoplankton samples was -160.9‰. The average and range of  $\epsilon_{\text{H}}$  values from the field samples under natural conditions are within the same range as  $\epsilon_{\text{H}}$

values derived from batch culture experiments (Caraco et al. 2010, Solomon et al. 2011) and other field studies (Doucett et al. 2007). There was a significant correlation between  $\epsilon_H$  and TP as well as between  $\epsilon_H$  and Chl-*a* (which is also correlated with TP). The positive relationship is likely due to a shift toward cyanobacterial dominance at higher TP concentrations. Cyanobacteria are rich in lipids, which are depleted in deuterium, thereby increasing the difference between  $\delta^2\text{H}_2\text{O}$  and the isolated phytoplankton sample (Hondula et al. 2013). Another potential hypothesis for the relationship between  $\epsilon_H$  and TP is that the samples were contaminated with nonphytoplankton material differentially along the TP gradient. If this were the case, we would expect  $\epsilon_C$  and  $\epsilon_H$  to be correlated, which they were not ( $p$ -value > 0.05).

Method A best reproduced measured  $\delta^{13}\text{C}_A$  values because the estimated and measured values were not significantly different. Method B also reasonably reproduced measured  $\delta^{13}\text{C}_A$  values because the slope of the regression between estimated and measured values among lakes was not significantly different from 1. For Method B, however, the intercept was significantly <0. Because the range of the data is far from the intercept, this bias is of less concern than a departure from unity in the slope. There is evidence that organic matter C:N changes during decomposition (e.g., Melillo et al. 1982), so that using the C:N value of live leaves in the watershed may present a bias in the calculation of the end member for the degraded terrestrial material in aquatic ecosystems. Because Method B was able to adequately reproduce the measured values, the introduced error is likely minimal.

Both methods A and B correct the isotope value of bulk POM using a mixing model with a live leaf end member; however, POM is a mixture of degraded leaves, soil organic matter, algal cells, and flocculated DOM. Regardless of actual POM composition, the calculated value of  $\delta^{13}\text{C}_A$  for Methods A and B would not equal the measured value of  $\delta^{13}\text{C}_A$  if the terrestrial end member values used in the calculation were not representative of the terrestrial portion of the POM pool. This is further evidence that live leaves are adequate for defining the terrestrial end member for mixing models.

Phytoplankton  $\delta^{13}\text{C}$  values produced by Method C were significantly different from measured values. Values of  $\epsilon_C$  have been reported to vary from 0 to 40‰ among systems (Bade et al. 2006). Variability in  $\epsilon_C$  makes it unlikely that the average value estimated from a number of systems would accurately represent  $\epsilon_C$  in any one system. Marty and Planas (2008) in their evaluation of methods for estimating  $\delta^{13}\text{C}_A$  also found that assigning  $\epsilon_C$  is inadequate; however, their method for estimating  $\epsilon_C$  differed from Method C evaluated here.

The 2 methods that Marty and Planas (2008)

recommend for estimating  $\delta^{13}\text{C}_A$  in food web studies are isolated phytoplankton samples (if available) and bulk POM  $\delta^{13}\text{C}$  corrected for composition using the ratio of algal C to total particulate C (C:Chl-*a*; similar to Methods A and B). Although the C:Chl-*a* correction method cannot be evaluated using our data, if the isolated phytoplankton  $\delta^{13}\text{C}$  samples from their study are compared to the C:Chl-*a* corrected  $\delta^{13}\text{C}$  (table 2 in Marty and Planas 2008) using the same linear regression evaluation methods as this study, the slope and intercept are not significantly different from unity. The C:Chl-*a* bulk POM correction method for  $\delta^{13}\text{C}_A$  presented in Marty and Planas (2008) performs as well as Method A for  $\delta^{13}\text{C}_A$  based on the criteria used in this analysis.

For estimating  $\delta^{15}\text{N}_A$ , both Methods A and B produced estimates that were significantly different from measured values. Only the new method proposed here (Method D), which accounts for the difference in C:N between phytoplankton and terrestrial material and applies that correction to the  $\delta^2\text{H}$  mixing model method, gave estimates of  $\delta^{15}\text{N}_A$  that were not significantly different from measured values. The use of C:N in Method B likely contributed to the lower mean absolute deviation for that method compared to Method A, although the overall estimates were still significantly different from the measured values.

## Recommendations

There was no substantial change in leaf pack H, C, or N isotope composition during the incubation experiments. These results suggest that leaves collected on land give a reasonable representation of the isotope composition of the terrestrial end member, especially because the  $\delta^2\text{H}$  value of fresh leaves includes the soluble compounds, which are likely the most labile portion of the terrestrial material in lakes. In many lakes, the terrestrial end member can also be determined using DOM because it is almost all terrestrial (Cole et al. 2011, Wilkinson et al. 2013). For example, excluding the eutrophic systems in 40 lakes studied in Wilkinson et al. (2013), mean  $\delta^2\text{H}$  of the remaining systems ( $n = 36$ ) was  $-127.9\text{‰}$  (SD 11.7), which is not significantly different from the terrestrial  $\delta^2\text{H}$  value ( $-129.5\text{‰}$ , SD 15.2) of freshly collected leaves used in that study. Although live leaves, surface DOM, and groundwater DOM (Solomon et al. 2011) have been demonstrated to be isotopically indistinguishable in some systems, further research is needed to see if this pattern holds for diverse systems.

Samples dominated by phytoplankton acquired either with net hauls or other isolating techniques are ideal for estimating the phytoplankton end member in mixing model studies. In most systems, however, isolating phyto-



plankton from POM is difficult (Hamilton et al. 2005). Based on our analyses, in systems where the POM pool is <70% terrestrial in origin we recommend using either of the bulk POM correction methods (A or B) because they adequately reproduced measured values of phytoplankton  $\delta^{13}\text{C}$ .

For Method A, the relationship between  $\epsilon_{\text{H}}$  and TP and the distribution of  $\epsilon_{\text{H}}$  values given here can also be used as prior information for estimating  $\delta^2\text{H}_{\text{A}}$  in a Bayesian framework in systems where only  $\delta^2\text{H}_2\text{O}$  is known. For systems >70% terrestrial, we recommend using Method C. Literature values of  $\epsilon_{\text{C}}$  can be used as an informed prior in a Bayesian framework if only one system was sampled. None of the methods we evaluated were able to adequately reproduce measured values of phytoplankton  $\delta^{15}\text{N}$ . Unless numerous basal resources are being evaluated, however,  $\delta^{15}\text{N}$  values are largely used to estimate trophic position (Post 2002), negating the need to estimate  $\delta^{15}\text{N}_{\text{A}}$ .

In this study, we examined different methods and assumptions for choosing the isotope end members for mixing models in aquatic ecosystems. Although we evaluated the terrestrial end member assumptions in both lotic and lentic systems, the phytoplankton calculation methods were only evaluated in north temperate lake ecosystems. Further research in a diversity of aquatic systems, both geographically and ecologically, is needed to better assess the utility of these methods across ecosystems.

## Acknowledgements

We thank Robert Johnson, Cal Buelo, and Jason Kurtzweil for assisting with field sampling and laboratory analysis. We also thank the University of Notre Dame Environmental Research Center staff for support. Two anonymous reviewers provided helpful comments to improve the manuscript. This work was funded by Sigma Xi Grant in Aid of Research (G20111015158252), University of Virginia Department of Environmental Sciences Exploratory Research Grant, and NSF DEB 0917719.

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