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

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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 (δ2H), carbon (δ13C), and nitrogen (δ15N) 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 δ2H, 0.0‰ for δ13C, and −0.1‰ for δ15N). 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 δ13C and δ15N of phytoplankton. One method, which corrected bulk particulate organic matter (POM) isotope values based on a δ2H mixing model, accurately predicted measured phytoplankton δ13C. 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 δ2H mixing model and correctly predicted measured phytoplankton δ15N. We recommend estimating phytoplankton δ13C and δ15N by correcting bulk POM using a δ2H mixing model, with the C:N modification proposed here for δ15N.

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 (13C/12C: δ13C), nitrogen (15N/14N: δ15N), and hydrogen (2H/1H: δ2H) 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 terrestrial 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 δ13C of leaves may not change appreciably, the change in δ18O 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 δD value because water generally has much more positive δD 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 δD 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, δ13C values can be calculated based on the discrimination (εc) between phytoplankton and the dissolved inorganic carbon (DIC) in the water. Hydrogen values can be calculated based on the discrimination (εh) between phytoplankton and water. In these cases, εc and εh 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 εc 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 δ13C 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 δ13C values as substitutes for phytoplankton δ13C. 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 δ13C 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 δ13C 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 δ13C values to calculate the terrestrial fraction of POM (ϕT) using a linear mixing model (see Table 1). The phytoplankton fraction (1 − ϕT) of POM was used to calculate the δ13C of phytoplankton (δ13Cϕ) based on the δ13C of POM. Francis et al. (2011) used a similar method of C:N ratios of phytoplankton and terrestrial material to derive ϕT. Both methods can also be used to derive the δ15N of phytoplankton by substituting δ15N POM and δ15N of terrestrial δ15N of phytoplankton. 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 δ13C and δ15N estimation methods presented in the literature. We also examined the variability in δD of phytoplankton and calculated εh for the 10 lakes because there are few published values of εh 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 δ13C and δ15N. 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. δX is used in equations where δ13C or δ15N values are used to calculate the respective phytoplankton end member. The parameter value for ρ was derived as described in the text.

<table>
<thead>
<tr>
<th>Method</th>
<th>Citation</th>
<th>Output</th>
<th>Equation 1</th>
<th>Equation 2</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cole et al. (2011)</td>
<td>δ13C_A, δ15N_A</td>
<td>[ \phi_t = \frac{(\delta^2H_{POM} - \delta^2H_A)}{(\delta^2H_t - \delta^2H_A)} ]</td>
<td>[ \delta X_A = \delta X_{POM} - \left( \frac{\phi_t \times \delta X_t}{1 - \phi_t} \right) ]</td>
<td>[ \delta^2H_A = \text{live leaves} ] [ \delta^2H_{POM} = \text{sampled} ]</td>
</tr>
<tr>
<td>B</td>
<td>Francis et al. (2011)</td>
<td>δ13C_A, δ15N_A</td>
<td>[ \phi_t = \frac{C:N_{POM} - C:N_A}{C:N_t - C:N_A} ]</td>
<td>[ \delta X_A = \delta X_{POM} - \left( \frac{\phi_t \times \delta X_t}{1 - \phi_t} \right) ]</td>
<td>[ C:N_A = \text{live leaves} ] [ C:N_{POM} = \text{sampled} ]</td>
</tr>
<tr>
<td>C</td>
<td>Mohamed and Taylor (2009)</td>
<td>δ13C_A</td>
<td>[ \varepsilon_C = \frac{\delta^{13}C_t - b}{m} \text{; where} ] [ \delta^{13}C_{POM} = \delta^{13}CO_2 \times m - b ]</td>
<td>[ \delta^{13}C_A = \delta^{13}CO_2 \times \varepsilon_C ]</td>
<td>[ \delta^{13}C_A = \text{sampled} ] [ \delta^{13}CO_2 = \text{sampled} ]</td>
</tr>
<tr>
<td>D</td>
<td>This study</td>
<td>δ15N_A</td>
<td>[ \phi_t = \frac{(\delta^2H_{POM} - \delta^2H_A)}{(\delta^2H_t - \delta^2H_A)} ]</td>
<td>[ \delta^{15}N_A = \frac{\delta^{15}N_{POM} - \left( \phi_t \times \rho \times \delta^{15}N_t \right)}{1 - \phi_t \times \rho} ]</td>
<td>[ \delta^{15}N_A = \text{live leaves} ] [ \delta^{15}N_{POM} = \text{sampled} ]</td>
</tr>
</tbody>
</table>

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 δ2H, δ13C, and δ15N 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 δ2H,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 δ2H analysis. The δ2H 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 δ2H 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 (α = 0.05) were used to examine the significance of changes in the leaf pack δ2H, δ13C, and δ15N 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 μM), back-rinsed, and dried at 60 °C. We obtained phytoplankton samples by net tows (80 μm 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 δ¹³C–DIC analysis. The equilibrium value of δ¹³C was calculated using δ¹³C–DIC value, pH, and temperature (Zhang et al. 1995). Water for δ¹⁵H analysis from each lake was collected and preserved as described above.

**Isotope analysis**

δ²H 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‰ for a hypothetical leaf with an actual value of −135‰ 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% (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 ±5% 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 δ²H (M. Caron, CPSIL, Mar 2013, pers. comm.). δ²H in water samples was analyzed using cavity ring-down laser spectroscopy; δ¹³C and δ¹⁵N isotope samples were analyzed at the University of Virginia. Typical reproducibility for δ¹³C and δ¹⁵N based on replicate analyses is >0.3‰. Steger Creek δ¹³C 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 δ²H, Pee Dee Belemnite (PDB) for δ¹³C, and atmospheric N₂ for δ¹⁵N.

**Phytoplankton discrimination factors**

Isotopic differences between a photosynthetic organism and the photosynthetic substrate can be expressed several ways. We used the discrimination factor, ε, determined for each of the 10 lakes using the following equation:

$$
\varepsilon = \frac{\delta^2H_{A} - \delta^2H_{O}}{1 + \left(\frac{\delta^2H_O}{1000}\right)},
$$

where δ²H₂O and δ²Hₐ are 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, ε, using δ¹³CO₂ in each lake instead of δ²H₂O, and δ¹⁵Cₜ instead of δ²Hₐ from the 10 lakes. A linear regression analysis was performed to determine if ε₂H or ε₁₃C 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 δ¹³C and δ¹⁵N 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 δ¹³Cₚ (Table 2). Method A from Cole et al. (2011) corrects the bulk POM δ¹³C value based on the terrestrial fraction of the POM calculated from a δ²H-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 δ¹³N, which was compared to δ¹⁵Nₚ (δ¹³N 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 ε₁₃C derived from a population of lakes in equation 1. The terrestrial end member isotope values (δ²Hₐ, δ¹³Cₚ, and δ¹⁵Nₚ) were from 81 leaf samples from Northern Highlands Lake District (Solomon et al. 2011). The terrestrial C:N value (C:Nₚ = 26.8) was estimated from 22 leaf samples from the same study. The value of C:Nₚ was the average of freshwater phytoplankton C:N values presented in Vuorio et al. (2006).

A new method (D) for estimating phytoplankton δ¹⁵N 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 δ²H, Method A) can provide a direct estimate of δ¹³Cₓ. 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).

<table>
<thead>
<tr>
<th>Lake</th>
<th>Phyto δ²H</th>
<th>Phyto δ¹³C</th>
<th>Phyto δ¹⁵N</th>
<th>POM δ²H</th>
<th>POM δ¹³C</th>
<th>POM δ¹⁵N</th>
<th>POM C:N</th>
<th>δ²H₂O</th>
<th>δ¹³CO₂</th>
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</thead>
<tbody>
<tr>
<td>Allequash</td>
<td>−226.8</td>
<td>−31.9</td>
<td>0.0</td>
<td>−194.4</td>
<td>−28.5</td>
<td>1.6</td>
<td>8.30</td>
<td>−66.4</td>
<td>−17.5</td>
</tr>
<tr>
<td>Big Lake</td>
<td>−209.3</td>
<td>−31.2</td>
<td>5.4</td>
<td>−206.8</td>
<td>−29.9</td>
<td>3.7</td>
<td>8.88</td>
<td>−60.3</td>
<td>−13.4</td>
</tr>
<tr>
<td>Big Muskellunge</td>
<td>−167.9</td>
<td>−19.8</td>
<td>4.1</td>
<td>−172.4</td>
<td>−22.3</td>
<td>2.4</td>
<td>12.58</td>
<td>−43.2</td>
<td>−10.9</td>
</tr>
<tr>
<td>Deadwood</td>
<td>−180.9</td>
<td>−33.9</td>
<td>1.2</td>
<td>−134.9</td>
<td>−29.3</td>
<td>0.7</td>
<td>10.63</td>
<td>−45.9</td>
<td>−17.5</td>
</tr>
<tr>
<td>Found</td>
<td>−236.7</td>
<td>−26.7</td>
<td>3.6</td>
<td>−175.9</td>
<td>−25.0</td>
<td>3.3</td>
<td>12.96</td>
<td>−49.7</td>
<td>−14.5</td>
</tr>
<tr>
<td>Inkpot</td>
<td>−200.9</td>
<td>−34.9</td>
<td>1.8</td>
<td>−171.0</td>
<td>−32.7</td>
<td>1.4</td>
<td>6.23</td>
<td>−62.1</td>
<td>−17.5</td>
</tr>
<tr>
<td>Little Arbor Vitae</td>
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<td>−24.0</td>
<td>3.4</td>
<td>−244.9</td>
<td>−25.7</td>
<td>0.2</td>
<td>10.97</td>
<td>−53.9</td>
<td>−10.7</td>
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<tr>
<td>Presque Isle</td>
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<td>4.8</td>
<td>−191.8</td>
<td>−27.1</td>
<td>3.9</td>
<td>8.77</td>
<td>−53.8</td>
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<tr>
<td>Sparkling</td>
<td>−193.2</td>
<td>−26.5</td>
<td>4.2</td>
<td>−172.3</td>
<td>−26.6</td>
<td>0.7</td>
<td>10.31</td>
<td>−48.2</td>
<td>−12.1</td>
</tr>
<tr>
<td>Tenderfoot</td>
<td>−222.7</td>
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<td>−186.9</td>
<td>−30.0</td>
<td>2.3</td>
<td>8.36</td>
<td>−64.4</td>
<td>−14.6</td>
</tr>
</tbody>
</table>

algal 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 $\delta^{15}C_A$ from Methods A–C and calculated $\delta^{15}N_a$ from Methods A, B, and D were compared to the directly measured $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^2H$ and percent mass loss. The average water $\delta^2H$ values in Peter Lake and Steger Creek were −45.1 and −36.4‰, respectively.

In both systems, there was a decline in leaf $\delta^2H$ (−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 $\delta^2H$ values among weeks (Fig. 1a; $F_{4,10} = 2.15, p = 0.15$). The average change in leaf $\delta^2H$ value after 8 weeks was +0.5‰. The average change in $\delta^2H$ 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 $\delta^{13}C$ for Peter Lake was −0.3 and −0.1‰, respectively (Fig. 1b). The changes in C isotope values during the incubation determined were insignificant ($F_{4,10} = 0.71, p = 0.60$), as were the changes in N isotope values ($F_{4,10} = 0.36, p = 0.83$). In contrast, Steger Creek leaf $\delta^2H$ values over the 2 months were significantly different (Fig. 1c; $F_{4,10} = 13.30, p < 0.001$). The change in $\delta^2H$ between weeks 0 and 8 was +1.8‰. The average change in $\delta^{15}N$ for Steger Creek was −0.1‰; however, none of the changes in $\delta^{15}N$ values were significantly different between weeks (Fig. 1d; $F_{4,10} = 1.3, p-value = 0.33$).

**Terrestrial transect sampling**

The $\delta^2H$ 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 $\delta^2H$ and distance from shore. The mean $\delta^2H$ value of benthic terrestrial fragments from Peter Lake was −144.1‰, ranging from −124.1 to −159.0‰. There were no correlations with depth or distance from shore and no significant difference in $\delta^2H$ between the leaf transect samples and the benthic terrestrial fragment samples (one-way ANOVA; $F_{1,10} = 1.3, p-value = 0.21$).
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...
measured values had a slope at unity, but the intercept was significantly different from 0 (Fig. 2b). Method C produced estimates of δ¹³C 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 δ¹⁵N with regression slopes significantly different from unity (Table 3; Fig. 2d and e). Method D was the only method that produced estimates of δ¹⁵N 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 δ²H values and are leached and degraded at varying rates, changes would be expected in the bulk level of the δ²H 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 (Ostrofsky 1997).

Although the pattern of initial rapid decline in leaf δ²H followed by a gradual increase was observed in both systems, the statistical significance of the differences between leaf δ²H varied between the 2 systems. In Peter Lake, there were no significant differences among leaf δ²H values from different sampling times. With only 11% of the leaf mass remaining after 8 weeks, the leaf δ²H was nearly equal to the initial values. While the leaf packs that incubated in Peter Lake for a year had slightly lower δ²H values than those from weeks 0–8 of the decomposition experiment, there was not a substantial difference in leaf δ²H.

Even if the largest difference in leaf δ²H 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 δ²H 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 δ²H to the terrestrial end member has an even smaller impact on the mixing model outcome. The consistency of the leaf δ²H 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 δ²H values over time, reflecting the rapid decline of leaf δ²H during the first week (Fig. 1c). The decrease of leaf δ²H 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 ε₂H calculated using isolated phytoplankton samples was −160.9‰. The average and range of ε₂H values from the field samples under natural conditions are within the same range as ε₂H.
values derived from batch culture experiments (Caraco et al. 2010, Solomon et al. 2011) and other field studies (Doucet et al. 2007). There was a significant correlation between $\varepsilon_{\text{H}}$ and TP as well as between $\varepsilon_{\text{H}}$ 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 $\delta^{2}H_{2}O$ and the isolated phytoplankton sample (Hondula et al. 2013). Another potential hypothesis for the relationship between $\varepsilon_{\text{H}}$ and TP is that the samples were contaminated with nonphytoplankton material differentially along the TP gradient. If this were the case, we would expect $\varepsilon_{\text{C}}$ and $\varepsilon_{\text{H}}$ to be correlated, which they were not (p-value > 0.05).

Method A best reproduced measured $\delta^{13}C_{\text{A}}$ values because the estimated and measured values were not significantly different. Method B also reasonably reproduced measured $\delta^{13}C_{\text{A}}$ 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 $\delta^{13}C_{\text{A}}$ for Methods A and B would not equal the measured value of $\delta^{13}C_{\text{A}}$ 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 $\delta^{13}C$ values produced by Method C were significantly different from measured values. Values of $\varepsilon_{\text{C}}$ have been reported to vary from 0 to 40‰ among systems (Bade et al. 2006). Variability in $\varepsilon_{\text{C}}$ makes it unlikely that the average value estimated from a number of systems would accurately represent $\varepsilon_{\text{C}}$ in any one system. Marty and Planas (2008) in their evaluation of methods for estimating $\delta^{13}C_{\text{A}}$ also found that assigning $\varepsilon_{\text{C}}$ is inadequate; however, their method for estimating $\varepsilon_{\text{C}}$ differed from Method C evaluated here.

The 2 methods that Marty and Planas (2008) recommend for estimating $\delta^{13}C_{\text{A}}$ in food web studies are isolated phytoplankton samples (if available) and bulk POM $\delta^{13}C$ 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 $\delta^{13}C$ samples from their study are compared to the C:Chl-$a$ corrected $\delta^{13}C$ (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 $\delta^{13}C_{\text{A}}$ presented in Marty and Planas (2008) performs as well as Method A for $\delta^{13}C_{\text{A}}$ based on the criteria used in this analysis.

For estimating $\delta^{15}N_{\text{A}}$, 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 $\delta^{2}H$ mixing model method, gave estimates of $\delta^{15}N_{\text{A}}$ 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 $\delta^{2}H$ 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 $\delta^{2}H$ of the remaining systems (n = 36) was $-127.9\%$ (SD 11.7), which is not significantly different from the terrestrial $\delta^{2}H$ value ($-129.5\%$, 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-
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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 δ13C.

For Method A, the relationship between εδ and TP and the distribution of εδ values given here can also be used as prior information for estimating δH2o in a Bayesian framework in systems where only δD H2O is known. For systems >70% terrestrial, we recommend using Method C. Literature values of εδ 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 δ15N. Unless numerous basal resources are being evaluated, however, δ15N values are largely used to estimate trophic position (Post 2002), negating the need to estimate δ15N.

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.

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