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A standard protocol for stable isotope analysis of zooplankton in aquatic food web research using mass balance correction models

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Abstract

Stable isotope analysis has become a crucial tool for aquatic food web ecologists, but a lack of methodological standardization hinders comparisons between studies. One methodological inconsistency in stable isotope food web research is the decision whether to extract lipids before stable isotope analysis. The depletion in zooplankton stable carbon isotope values ($\delta^{13}C$) due to fatty acid content and the accuracy of mathematical correction models designed to predict this depletion were examined for a range of zooplankton species from nine lakes of diverse size and productivity. Large differences of up to 5‰ observed between $\delta^{13}C$ values of nonextracted and lipid-extracted zooplankton samples correlated with zooplankton fatty acid content. A mass balance $\delta^{13}C$ correction model for fatty acid content using atomic C : N ratios and directly measured $\delta^{13}C$ values of fatty acids accurately predicted ($R^2 = 0.95$) lipid-extracted $\delta^{13}C$ values for both copepod and cladoceran zooplankton. Researchers should use mass balance lipid corrections as an efficient method to eliminate bias in comparisons of zooplankton and fish $\delta^{13}C$ values and allow their results to be more easily compared with other studies.

The stable isotope composition of organic carbon ($^{13}C : ^{12}C$) and nitrogen ($^{15}N : ^{14}N$) can be used to provide detailed descriptions of aquatic food web structure and models of fish and zooplankton diet (Kling and Fry 1992; Keough et al. 1996; Vander Zanden et al. 1999). The stable carbon isotope signature ($\delta^{13}C$) of a consumer reflects its source of dietary carbon (Peterson and Fry 1987; Post 2002), whereas the stable nitrogen isotope value ($\delta^{15}N$) can describe the trophic position of an organism (Minagawa and Wada 1984; Post 2002). Therefore, the combined measurements of $\delta^{13}C$ and $\delta^{15}N$ can be used to examine how the basal carbon source of a food web is successively transferred to higher trophic levels.

Although stable isotope analysis has led to significant advances in understanding ecological and biogeochemical processes in lake systems, there is a lack of standard protocols for sample preparation, preservation, and analysis, which can make data interpretation and cross-study comparisons difficult and confusing (Pinnegar and Polunin 1999; Feuchtmayr and Grey 2003; Kiljunen et al. 2006). Inconsistencies include the type of tissue used for analysis (Pinnegar and Polunin 1999), allowance for zooplankton gut clearance (Feuchtmayr and Grey 2003), and sample acidification (Bunn et al. 1995). Although some issues have been addressed, thorough investigations of other aspects of sample preparation are needed to establish a standard protocol. A recently debated issue is the need to extract lipids from zooplankton and fish tissue before stable isotope analysis (Kiljunen et al. 2006; Murry et al. 2006; Sweeting et al. 2006).

Variation in lipid content can have significant effects on the $\delta^{13}C$ value of organisms, thereby affecting food web interpretation (Matthews and Mazumder 2005; Murry et al. 2006; Sweeting et al. 2006). In most freshwater zooplankton and planktivorous fish, lipids are present as

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fatty acids in triglycerides and phospholipids (Henderson and Tocher 1987; Olsen 1999). Fatty acids in primary and secondary consumers are depleted in ^{13}C by up to 9‰ relative to whole tissues of an organism because of the mechanism of their biosynthesis (DeNiro and Epstein 1977; Abrajano et al. 1994; Veefkind 2003). Thus samples with high fatty acid content will be biased toward more depleted $\delta^{13}C$ values. Zooplankton fatty acid content varies seasonally, sometimes by more than a factor of five, in relation to food abundance and reproductive cycle (Vanderploeg et al. 1992; Arts et al. 1993; Smyntek 2006). In addition, the range in fatty acid content between different species can exceed an order of magnitude (Persson and Vrede 2006; Smyntek 2006). Therefore, eliminating the effects of variable fatty acid content among zooplankton on $\delta^{13}C$ values is critical for a more accurate interpretation of data from stable isotope studies of aquatic food web structure and planktivorous fish diet.

Comparisons of lipid-extracted $\delta^{13}C$ values ($\delta^{13}C_{ex}$) of organisms would prevent bias caused by variation in fatty acid content among different species or individuals. However, lipid extraction can cause significant increases in the $\delta^{15}N$ values of whole zooplankton and fish tissue samples relative to nonextracted replicates (Murry et al. 2006; Smyntek 2006; Sweeting et al. 2006). Therefore, such enrichment in ^{15}N caused by lipid extraction requires that a separate, nonextracted portion of the sample be analyzed for accurate measurement of its $\delta^{15}N$ value. This is an expensive and time-consuming process that also requires a greater amount of sample.

Mathematical lipid correction models are alternatives to lipid extraction that account for the effects of lipid storage on $\delta^{13}C$ values in organisms (McConnaughey and McRoy 1979; Fry et al. 2003; Sweeting et al. 2006). These correction models are derived from relations between the $\delta^{13}C$ values of lipid and nonlipid components of a sample and its lipid content. Atomic C : N ratios are often a good proxy for lipid content (Sterner and Elser 2002; Sweeting et al. 2006), and they can be measured simultaneously with the analysis of the $\delta^{13}C$ and $\delta^{15}N$ values of a sample. The different models for obtaining $\delta^{13}C_{ex}$ from atomic C : N ratios are the lipid normalization model (McConnaughey 1978), which assumes that the low content of carbohydrates in animals is constant and that organisms with low lipid content (C : N of 4.0) will exhibit no difference between lipid-extracted and nonextracted $\delta^{13}C$ values. The second mass balance correction model assumes that lipids and proteins are the only two sources of organic carbon and protein the only source of organic nitrogen in a sample (Fry et al. 2003). The ability of this mass balance model to accurately predict $\delta^{13}C_{ex}$ for fish muscle tissue has been confirmed (Sweeting et al. 2006). Therefore, if the mass balance correction model can be applied to the analysis of zooplankton samples, this procedure can be adopted as a standard method for analysis of zooplankton and fish food web interactions that eliminates bias caused by variable lipid content and allows simultaneous measurement of the $\delta^{13}C$ and $\delta^{15}N$ values of a sample.

The objectives of the present study were to address two main questions. First, what is the magnitude of the effect of lipid extraction on $\delta^{13}C$ values ($\Delta^{13}C_{ex\text{-}non}$) for different zooplankton species and what implications does extraction have on interpretation of food web structure? Second, can $\delta^{13}C_{ex}$ be predicted accurately from atomic C : N ratios of zooplankton using a mass balance correction or lipid normalization model? To answer these questions, 120 samples encompassing a variety of zooplankton species were collected from nine lake systems of diverse sizes and productivities from two geographically distinct regions (Great Lakes, U.S.A., and English Lake District, U.K.) and analyzed for their fatty acid content, pre- and postlipid-extracted $\delta^{13}C$ values, and pre- and postlipid-extracted atomic C : N ratios. Rather than assuming the stable isotope composition of fatty acids from literature studies of unrelated organisms, as has been done in previous studies (e.g., McConnaughey and McRoy, 1979; Matthews and Mazumder, 2005; Kiljunen et al. 2006), we directly measured the $\delta^{13}C$ values of individual fatty acids for a subset of these samples.

Materials and methods

Field sampling—Zooplankton were collected over the summer season from nine lakes of varying size and productivity (Table 1). Zooplankton from nearshore and offshore sites in Lakes Michigan, Erie, Ontario, and Champlain were collected monthly with 50-μm- (30-cm diam.) and 500-μm- (50-cm diam.) mesh Wisconsin high-efficiency plankton nets in vertical tows of the entire water column during the summers of 2002 and 2003. Samples were immediately frozen on a nylon mesh with dry ice before being transported to the laboratory and stored at $-80^{\circ}C$. Zooplankton collected from the Lake District (U.K.) lakes in the summer of 2005 (Grasmere, Derwent Water, Crummock Water, and the north and south basins of Windermere) were collected biweekly at deep, well-established sampling sites with vertical tows of the entire water column using a 120-μm-mesh net (30-cm diam.). These zooplankton were allowed to remain in lake water in a cool place (generally for 2 to 4 h, but never more than 24 h) to allow for gut clearance before being transferred onto a nylon mesh and frozen for storage. All samples were thawed, sorted to genera or species using taxonomic keys, frozen again, and then lyophilized at $-55^{\circ}C$ before stable isotope and fatty acid analysis. The four cladocerans analyzed included the herbivore *Daphnia* spp. and the predators *Cercopagis pengoi* (Ostroumov), *Bythotrephes longimanus* (Leydig), and *Leptodora kindti* (Focke; Balcer et al. 1984; Thorp and Covich 1991; Scourfield and Harding 1966). The calanoid copepods from the Great Lakes and Lake Champlain were largely represented by *Diaptomus* sp. and some *Limnocalanus* sp., whereas the dominant cyclopoid copepods were typically *Diacyclops thomasi* (Forbes) and *Mesocyclops edax* (Forbes; Balcer et al. 1984). In the English lakes, calanoid copepods were identified and separated to species, which included *Eudiaptomus gracilis* (Sars) and *Diaptomus laticeps* (Sars), while all of the individually separated cyclopoid copepods were *Cyclops strenuus abyssorum* (Sars; Harding and Smith 1974).

Table 1. Location and sampling dates for zooplankton sample collection from lakes in North America and the Lake District, U.K.

Lake name	Calanoid copepods $N=38$	Cyclopoidcopepods $n=15$	*Daphnia* $n=26$	*Bythotrephes* $n=18$	*Cercopagis* $n=12$	*Leptodora* $n=11$
Lake Michigan	Jun 02, Jul 02, Aug 02, Sep 02, May 03, Jun 03, Jul 03 × 2, Aug 03, Sep 03 × 2	May 03, Jul 03, Sep 03	Sep 02, Jul 03, Aug 03, Sep 03	Jul 02, Aug 02, Sep 02, Jul 03, Aug 03, Sep 03	Aug 02, Sep 02, Jul 03 Aug 03 × 2	Sep 03
Lake Erie	Jul 02, Oct 02, Jul 03	Jul 02	Jul 02, 10/02	Jul 03, Jul 02 × 2	NA	Aug 02
Lake Ontario	Jun 02 × 2,702, Aug 02, May 03, Aug 03, Sep 03	Jun 02, May 03, Aug 03	Sep 02, Aug 03, Sep 03, Oct 03	NA	Jul 02 × 2, Aug 02 × 2, Sep 02, Aug 03, Oct 03	NA
Lake Champlain	Jul 02, Jul 03	NA	Jul 02 × 2, Jul 03	NA	NA	Jul 03
*Windermere North Basin	26 Jul 05 × 2, 08 Aug 05 × 2, 22 Aug 05 × 2	26 Jul 05, 08 Aug 05, 2 Aug 05	12 Jul 05, 26 Jul 05, 08 Aug 05, 22 Aug 05	12 Jul 05, 26 Jul 05, 08 Aug 05, 22 Aug 05	NA	26 Jul 05, 08 Aug 05, 22 Aug 05
*Windermere South Basin	12 Jul 05, 26Jul 05, 09 Aug 05, 22 Aug 05	12 Jul 05, 09 Aug 05, 22 Aug 05	12 Jul 05, 09 Aug 05, 22 Aug 05	12 Jul 05, 09 Aug, 22 Aug 05	NA	12 Jul 05, 22 Aug 05
*Derwent Water	29 Jun 05, 13 Jul 05, 27 Jul 05, 10 Aug 05, 25 Aug 05	NA	16 Jun 05, 29 Jun 05	NA	NA	10 Aug 05, 25 Aug 05
*Grasmere	NA	11 Jul 05	11 Jul 05, 25 Jul 05, 08 Aug 05	25 Jul 05, 08 Aug 05	NA	08 Aug 05
*Crummock Water	19 Jul 05	19 Jul 05	19 Jul 05	19 Jul 05	NA	NA

* Dates are listed Mmm yy or dd Mmm yy; "× 2" indicates that two separate samples, either from distinct nearshore and offshore sampling sites or of two separate, individually sorted species, were collected on that date; NA indicates the sample was not available because of absence or insufficient numbers of the organism in the water column. The number of zooplankton samples is indicated as "*n*" below each zooplankton category.

Fatty acid analysis—Fatty acid extraction and derivatization procedures were similar to those of Wacker and von Elert (2001). In brief, 0.15–2 mg of dried zooplankton tissue were extracted three times in dichloromethane : methanol (2 : 1). Residual solvent was removed from the extracted zooplankton sample with a gentle stream of nitrogen, and the dried, extracted zooplankton were stored in the freezer before stable isotope analysis. Total organic extracts were then either saponified (6% potassium hydroxide in methanol for 60 min at 70°C) to isolate fatty acids and subsequently esterified with methanolic hydrochloric acid (Sigma, 60 min at 60°C), or they were esterified with methanolic hydrochloric acid directly and then silylated with bis(trimethylsilyl)-trifluoroacetamide (Sigma; 60 min at 60°C) to protect any alcohol groups. Gas chromatography (GC) analysis was performed on a Hewlett Packard 5890 using either an Omegawax (Supelco) or a DB-5 (J&W Scientific) column (both 30 m, i.d. 0.25 mm, film thickness 0.25 μm). The oven temperature program for the Omegawax column started at 60°C for 1 min, increased to 160°C at 15°C min^{-1}, then increased to 265°C at 5°C min^{-1} and remained there for 25 min. The split/splitless injector and detector temperatures were both 250°C, and the column flow was 1.3 mL min^{-1} of helium. The DB-5 column temperature program began at 60°C for 1 min, increased to 140°C at 15°C min^{-1}, and then increased to 300°C at 4°C min^{-1} and remained there for 15 min. The split/splitless injector and detector temperatures were both 250°C and the column flow was 2.6 mL·min^{-1} of helium. Final derivatized extract volumes were between 50 and 2,000 μL, and the injection volume was always 1 μL. Fatty acids were identified using standard mixtures (Supelco) by GC or by GC-mass spectrometry (MS) (Shimadzu QP 5050) operating in electron-impact mode, and concentrations were determined by GC using an internal standard (nonadecanoic acid) that was added to each sample before saponification or esterification. The instrument detection limit was 2 ng on the GC column, and error in measurement was 1–12%.

Bulk stable isotope analysis—Samples of nonextracted and lipid-extracted zooplankton were prepared for stable isotope analysis by weighing 80 to 600 μg of dried material into preweighed tin boats. Samples were combusted in a Costech ECS 4010 elemental analyzer coupled to a Thermo-Finnigan Delta XL Plus isotope ratio mass spectrometer (IRMS) or a Carlo Erba NC 2500 elemental analyzer coupled to a Finnigan MAT 252 IRMS. Both instruments were run in continuous flow mode, allowing simultaneous measurement of stable carbon isotope values and atomic C : N ratios based on calibration with an acetanilide standard. The weight percentages of carbon and nitrogen outputted from the instrument were converted to atomic C : N ratios. Stable carbon isotope values are reported in the standard format in parts per thousands (per mil, ‰) relative to Vienna Pee Dee Belemnite. The accuracy and precision of measurement was independently verified using National Institute of Standards and Technology (NIST) Australian National University (ANU) sucrose ($\delta^{13}C = -10.5 \pm 0.2$ [$n = 39$]), National Bureau of Standards (NBS) 22 oil (-29.7 ± 0.2 [$n = 25$]), and NIST 1587 peach leaves (-25.8 ± 0.1 [$n = 85$]). Daily precision of the instrument was verified by repeated analysis of internal laboratory standards including acetanilide (-29.9 ± 0.2 [$n = 32$]), fish muscle tissue (-18.1 ± 0.2 [$n = 22$]), and plant tissue (-28.0 ± 0.2 [$n = 18$]), which were analyzed throughout the sample runs. Samples were analyzed in duplicate or triplicate when sufficient material was available, and differences or standard deviations exceeded 0.8‰ for only 1% of the samples and averaged 0.2‰ ± 0.2‰ for all samples.

Compound-specific stable isotope analysis of fatty acids—The stable carbon isotope values of individual fatty acids, analyzed as their methyl esters, were directly measured using a Finnigan TraceGC Ultra GC system with a Finnigan GC-C-III combustion interface connected to the Thermo-Finnigan Delta XL Plus IRMS. An Omegawax (30 m, i.d. 0.32 mm, film thickness 0.25 μm; Supelco) or an RTX-1 (60 m, i.d. 0.32 mm, film thickness 0.5 μm; Restek) column was used for chromatographic separation. The temperature program for the Omegawax column began at 60°C for 1 min, increased to 160°C at 15°C min^{-1}, then increased to 260°C at 5°C min^{1} and remained there for 15 min. The injector temperature was 240°C and column flow was 1.4 mL min^{-1} of helium. The RTX-1 column temperature program began at 60°C for 1.25 min, increased to 160°C at 15°C min^{-1}, and then increased to 280°C at 4°C min^{-1} and remained there for 9 min. The injector temperature was 250°C, and column flow was 2.3 mL min^{-1} of helium for this second column method. For both column methods, the combustion furnace was set at 950°C. Samples were measured in triplicate when possible, and stable carbon isotope values were calculated relative to an internal standard (nonadecanoic acid) of known isotope composition to correct for the addition of a methyl group (Abrajano et al. 1994). The precision for this method was typically 0.5‰ on the basis of repeated analysis of internal and external nonadecanoic acid standards. A weighted average based on relative abundance of the different fatty acids was then calculated to obtain an average value of $\delta^{13}C_{\text{fatty acid}}$ for comparison with $\delta^{13}C_{ex}$. As a quality-assurance measure, $\delta^{13}C_{bulk}$ was recalculated from $\delta^{13}C_{\text{fatty acid}}$ and $\delta^{13}C_{ex}$ using the measured fatty acid content per mass of organic carbon to determine the relative contribution of each $\delta^{13}C$ value in a two-source mixing model. Calculated values of $\delta^{13}C_{bulk}$ were within 0.6‰ of directly measured values for over 85% of the samples.

Mathematical correction models—The lipid normalization model is shown in Eqs. 1 and 2; the constants in these equations are derived from the model assumptions (McConnaughey 1978).

$$L = \frac{93}{1 + (0.246[\text{C} : \text{N}_{\text{bulk}}] - 0.775)^{-1}} \tag{1}$$

$$\delta^{13}C_{ex} \approx \delta^{13}C' = \delta^{13}C_{bulk} + D\left(-0.2068 + \frac{3.9}{1 + 287.1/L}\right) \tag{2}$$

This model uses atomic C : N ratios of the bulk, nonextracted sample ($C : N_{bulk}$) to calculate a lipid factor (L) that is then used to calculate $\delta^{13}C'$. $\delta^{13}C'$ is essentially equivalent to $\delta^{13}C_{ex}$ because this model assumes that proteins and carbohydrates have the same $\delta^{13}C$ value, which is generally accepted (Hayes 2001). In Eq. 2, D is the average difference between $\delta^{13}C$ values of protein and lipids in the sample, which is often estimated as 6‰ (Leggette 1998), and $\delta^{13}C_{bulk}$ is the value of the nonextracted sample.

In the mass balance model (Eqs. 3 and 4; Fry et al. 2003), values of D and the atomic C : N of protein for the samples are used in conjunction with the atomic $C : N_{bulk}$ to determine the depletion in $\delta^{13}C$ due to fatty acids, which is equivalent to the $\delta^{13}C$ value of the fatty acid-extracted sample minus $\delta^{13}C_{bulk}$. This difference, which is denoted as $\Delta^{13}C_{ex\text{-}non}$, is combined with $\delta^{13}C_{bulk}$ to calculate $\delta^{13}C_{ex}$.

$$\delta^{13}C_{ex} = \delta^{13}C_{bulk} + \Delta^{13}C_{ex-non} \quad (3)$$

$$\Delta^{13}C_{ex-non} = D\left(\frac{C : N_{bulk} - C : N_{ex}}{C : N_{bulk}}\right) \quad (4)$$

Thus the mass balance model determines the effect of lipid content on the nonextracted $\delta^{13}C$ value of the sample on the basis of the amount of carbon from lipids that increases the atomic C : N of the sample above the atomic C : N of protein.

Statistical analysis—All averages are reported with the standard deviation of the mean. Statistical tests including t-tests, linear regression, and one-way analysis of variance (ANOVA) with Tukey's family error rate were performed using MiniTab Release 14 software. The residuals from these statistical analyses were examined for normality and heteroscedasticity. Data that were not normally distributed or had unequal variances were transformed using a Box-Cox transformation before performing statistical tests. Comparisons were considered statistically significant at $\alpha = 0.05$.

Results

Lipid extraction significantly changed the stable carbon isotope values of zooplankton, and these changes were correlated to fatty acid content and atomic C : N ratios. The stable carbon isotope values of lipid-extracted samples ($\delta^{13}C_{ex}$) were significantly enriched in ^{13}C compared with those of nonextracted samples ($\delta^{13}C_{bulk}$; paired t-test, $p < 0.001$ for all samples, shown graphically for copepods in Fig. 1). The difference between $\delta^{13}C_{ex}$ and $\delta^{13}C_{bulk}$, denoted $\Delta^{13}C_{ex\text{-}non}$, ranged from 0‰ to +5.0‰ for some copepods samples ($n = 120$). The average value of $\Delta^{13}C_{ex\text{-}non}$ was not significantly different among the four types of cladocerans (*Daphnia* +1.7‰ ± 0.5‰, *Bythotrephes* +1.5‰ ± 0.5‰, *Cercopagis* +1.7‰ ± 0.4‰, and *Leptodora* +1.5‰ ± 0.4‰; one-way ANOVA, Tukey's family error rate, $F = 1.41$, $p = 0.249$, df = 66). $\Delta^{13}C_{ex\text{-}non}$ was significantly correlated with fatty acid content for copepods (Fig. 2A;

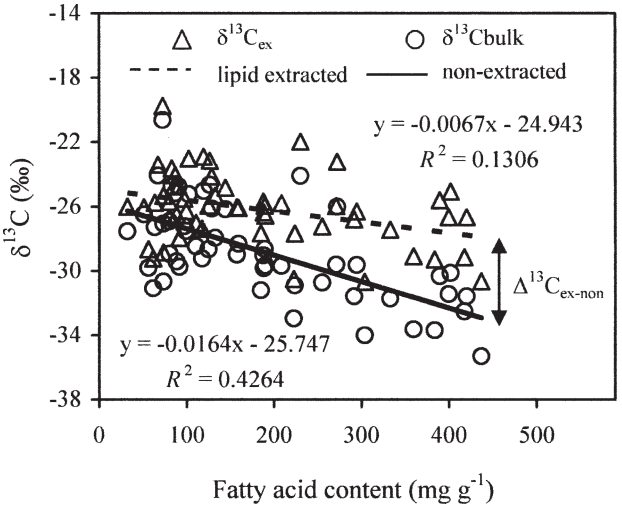

Fig. 1. The $\delta^{13}C$ values of lipid-extracted ($\delta^{13}C_{ex}$) and nonextracted ($\delta^{13}C_{bulk}$) zooplankton as a function of fatty acid content of copepods ($n = 53$). $\Delta^{13}C_{ex\text{-}non}$ is the difference between $\delta^{13}C_{bulk}$ and $\delta^{13}C_{ex}$, and is approximated as the difference between the lipid-extracted (dashed) and nonextracted (solid) linear regression lines.

$R^2 = 0.77$, $F = 176.20$, $p < 0.001$, df = 52) and cladocerans (Fig. 2B; $R^2 = 0.15$, $F = 11.62$, $p = 0.001$, df = 66), though the latter relation was considerably weaker. The small range (+0.4–2.5‰) and relatively low coefficient of variation (28.3%) of $\Delta^{13}C_{ex\text{-}non}$ for cladocerans compared with copepods were consistent with trends in fatty acid content and atomic C : N ratios for these distinct zooplankton groups.

Cladocerans contained a modest total fatty acid content (84 ± 22 mg g^{-1} dry weight) ranging from 41 to 140 mg g^{-1} (Fig. 2B). Fatty acid contents of the four individual cladoceran genera (*Daphnia, Bythotrephes, Cercopagis*, and *Leptodora*) were not significantly different from each other (one-way ANOVA, as above, $F = 1.60$, $p = 0.199$, df = 66), and therefore they were pooled together for comparison with the copepods. Calanoid (191 ± 124 mg g^{-1}) and cyclopoid (171 ± 103 mg g^{-1}) copepods both contained significantly higher amounts of fatty acids compared with the cladocerans (one-way ANOVA, as above, $F = 21.16$, $p < 0.001$, df = 119) and spanned much larger ranges (calanoids 51–420 and cyclopoids 32–400 mg g^{-1}; Fig. 2A). Variation in fatty acid content for the same calanoid copepod species (*Eudiaptomus gracilis*) exceeded a factor of six between different lakes within the Lake District (U.K.). Within a single lake (Windermere North Basin, U.K.), fatty acid content varied by nearly a factor of three for *Cyclops strenuus abyssorum* over a 2-month summer sampling period.

Calanoid and cyclopoid copepods also exhibited larger ranges in their atomic C : N ratios, 4.8–17.8 (average = 7.9 ± 3.7) and 5.2–12.1 (average = 7.5 ± 2.0), respectively, compared with those of cladocerans, 4.7–7.5 (average = 5.6 ± 0.6), reflecting differences in fatty acid content. The natural log of the atomic C : N ratio was significantly correlated with fatty acid content for copepods (Fig. 3A; $R^2 = 0.87$, $F = 350.39$, $p < 0.001$, df = 52), but not for

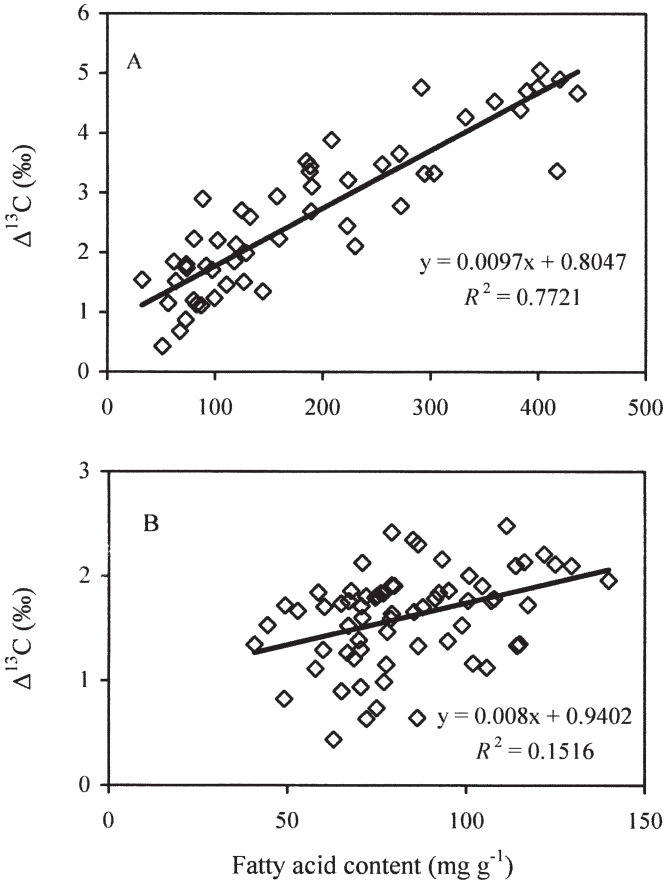

Fig. 2. The difference in stable carbon isotope composition between lipid-extracted and nonextracted $\delta^{13}C$ values ($\Delta^{13}C_{ex\text{-}non}$) of individual samples as a function of fatty acid content of (A) calanoid and cyclopoid copepods (n = 53) and (B) cladocerans (n = 67). Note the differences in the scales between panels A and B.

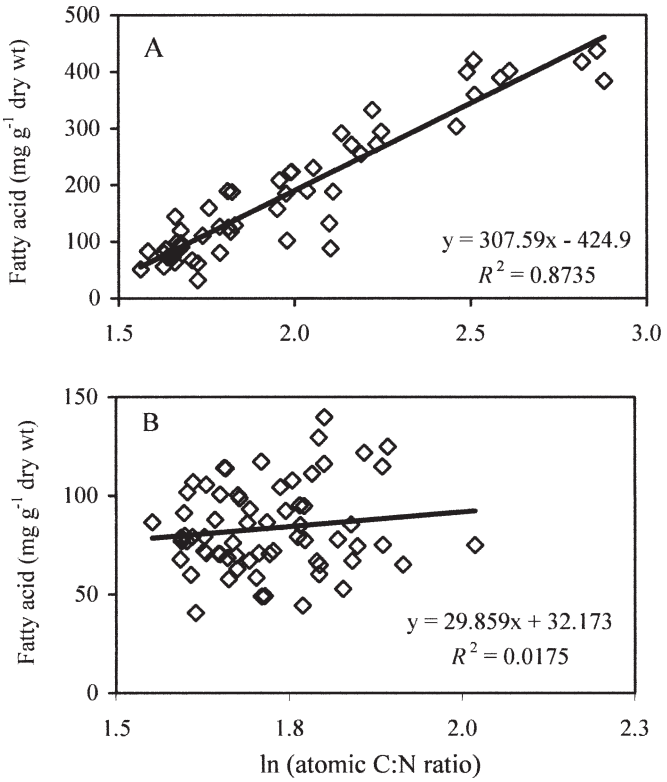

Fig. 3. Fatty acid content (mg g^{-1}) versus the natural log of atomic C : N ratio of (A) calanoid and cyclopoid copepods (n = 53) and (B) cladocerans (n = 67). Note the difference in scales between A and B.

cladocerans (Fig. 3B; R^2 = 0.02, F = 1.13, p = 0.29, df = 66). This logarithmic relation between fatty acid content and atomic C : N ratio is a reasonable approximation over the range of atomic C : N generally observed for zooplankton (less than ~18). In contrast to the range of atomic C : N in the nonextracted samples, the average zooplankton atomic C : N ratios subsequent to lipid extraction were quite uniform, supporting the assumption that material left after extraction was dominated by proteins. These average lipid-extracted atomic C : N values (C : N_{ex}) ranged from 4.0 ± 0.2 for calanoids to 4.5 ± 0.4 for daphnids, indicating that there was low variation both within and among different types of zooplankton.

Direct measurement of the $\delta^{13}C$ values of individual fatty acids by GC-C-IRMS showed that they were consistently depleted in ^{13}C relative to the $\delta^{13}C$ values of whole zooplankton, with differences as great as 12‰ for some major fatty acids. The average difference between the $\delta^{13}C$ value of combined fatty acids ($\delta^{13}C_{fatty\ acid}$) and the $\delta^{13}C$ value of the nonextracted zooplankton ($\delta^{13}C_{bulk}$) was 4.2‰ ± 1.3‰ (n = 30). The differences (D) between $\delta^{13}C_{fatty\ acid}$ and $\delta^{13}C$ of extracted zooplankton samples ($\delta^{13}C_{ex}$) varied among species and between lakes (Table 2), ranging from 4.3‰ to 8.8‰, with an average of 6.3‰ ± 1.3‰. Zooplankton from Lake Michigan had significantly higher values of D than those of Grasmere and the north and south basins of Windermere (one-way ANOVA, as above, F = 15.09, $p < 0.001$, df = 29). Within the three English lakes, copepods had D values that were significantly larger than those of cladocerans by an average of 1.1‰ (two-sample t-test, p = 0.002). However, in Lake Michigan there was no significant difference in D values between these two categories of zooplankton (two-sample t-test, p = 0.244). Overall, the copepods had a slightly higher average value of D than the cladocerans (6.5‰ ± 0.9‰, n = 12 and 6.1‰ ± 1.5‰, n = 18, respectively), but this difference was not significant (two-sample t-test, p = 0.176).

The two mathematical correction models differed in their ability to predict $\Delta^{13}C_{ex\text{-}non}$ for zooplankton, particularly samples with high atomic C : N ratios (shown for copepods, Fig. 4). The mass balance correction model accurately predicted $\delta^{13}C_{ex}$ for both copepods and cladocerans from their bulk $\delta^{13}C$ signatures and atomic C : N ratios (Fig. 5). The average measured values of D = 6.3‰ ± 1.3‰ (n = 30) and C : N_{ex} = 4.2 ± 0.4 (n = 120) for all zooplankton were used as the two constants in the mass balance model, and predicted values of $\delta^{13}C_{ex}$ were not significantly different from observed values (paired t-test, p = 0.064). The average absolute difference between the mass balance model predicted and observed values of $\delta^{13}C_{ex}$ was 0.5‰ ± 0.4‰, and it exceeded 1.0‰ for less than 8% of the total

Table 2. Difference (D [‰]) between weighted averaged values of $\delta^{13}C_{fatty\ acids}$ and $\delta^{13}C_{ex}$ (lipid-extracted) for zooplankton from four lakes. Listed as the average (bolded, plus or minus the standard deviation of the mean*) followed by the range in parentheses and the number of samples, n.

Lake name	Calanoid copepods	Cyclopoid copepods	*Daphnia*	*Bythotrephes*	*Cercopagis*
Lake Michigan	**7.8**‰±**0.5**‰ (7.5–8.3) n=3	NA	**7.2**‰±**0.8**‰ (6.9–8.1) n=3	**7.3**‰±**0.6**‰ (6.7–7.9) n=3	**8.2**‰±**1.3**‰* (7.5, 8.8) n=2
Windermere North Basin	**6.1**‰±**0.6**‰ (5.7–6.9) n=3	5.3‰ n=1	**4.7**‰±**0.7**‰ (4.3–5.5) n=3	**4.8**‰±**0.6**‰ (4.3–5.4) n=3	NA
Windermere South Basin	6.3‰ n=1	**5.9**‰±**0.3**‰ (5.6–6.3) n=3	NA	NA	NA
Grasmere	NA	7.3‰ n=1	**4.4**‰±**0.1**‰* (4.4, 4.5) n=2	**6.3**‰±**1.2**‰* (5.7, 6.8) n=2	NA

* Indicates the difference between duplicate samples rather than standard deviation. NA indicates the sample was not available because of insufficient numbers of the organism in the water column or because of limitations on the number of stable carbon isotope measurements of individual fatty acids that could be performed.

Fig. 4. Observed (crosses) and predicted values of $\Delta^{13}C_{ex\text{-}non}$ from mass balance (MB) and lipid normalization (LN) models versus atomic C : N ratio of copepods (n = 53) for different values of D (the average difference between $\delta^{13}C_{ex}$ and $\delta^{13}C_{fatty\ acid}$) and a constant value of $C:N_{ex}$ = 4.2.

120 samples. Predicted values of $\delta^{13}C_{ex}$ were neither consistently depleted nor enriched in ^{13}C relative to observed values. The slope and y-intercept of the regression analysis of model predicted versus measured values of $\delta^{13}C_{ex}$ were not significantly different from 1 and 0, respectively, over a large range of values (Fig. 5; t-tests, p = 0.617, and p = 0.486 for the slope and y-intercept, respectively).

In contrast to the mass balance model, the lipid normalization model often failed to accurately predict values of $\delta^{13}C_{ex}$ for some zooplankton using the same average value of D. An underestimation of $\Delta^{13}C_{ex\text{-}non}$, particularly for copepods (Fig. 4), resulted in predicted values of $\delta^{13}C_{ex}$ that were significantly more depleted in ^{13}C compared with observed values by 0.4‰ ± 0.6‰ (paired t-test, $p < 0.001$). The average absolute difference between observed values of $\delta^{13}C_{ex}$ and those predicted by lipid normalization was 0.6‰ ± 0.5‰, and this difference exceeded 1.0‰ in nearly 18% of the samples, most of them copepods with large atomic C : N ratios.

Fig. 5. The $\delta^{13}C_{ex}$ values predicted from the mass balance model versus observed $\delta^{13}C_{ex}$ (lipid-extracted values) for zooplankton (n = 120).

The mass balance model was sensitive to the value of D used to calculate $\delta^{13}C_{ex}$, especially for copepods with high atomic C : N ratios (Fig. 4). When the average value of D (6.3‰) used in the mass balance model was substituted with individually measured values of D for 30 samples for which it was available, the absolute difference between observed and mass balance model-predicted values of $\delta^{13}C_{ex}$ decreased significantly, from 0.6‰ to 0.3‰ (paired t-test, $p < 0.001$). The latter value begins to approach the analytical error of most IRMS instruments. In addition, when using individually measured D values in the mass balance model, only 1 of 30 samples had a predicted value $\delta^{13}C_{ex}$ greater than 1.0‰ different from the corresponding measured value.

Discussion

Stable isotope analysis has increasingly demonstrated its utility as an effective means for examining food web structure in many ecosystems, especially those that are otherwise difficult to study by conventional means (Ponsard and Arditi 2000). However, there have been appeals for more careful experimentation to better define both mechanisms of stable isotope fractionation in organisms and the limits of its usefulness in certain settings (Gannes et al. 1997). The present work quantifies depletions in $\delta^{13}C$ values of zooplankton due to their fatty acid content, which has important consequences for interpretation of aquatic food web structure, and demonstrates the ability of a mathematical mass balance correction model to accurately predict these depletions. The end result is a simple, cost-effective, and efficient way to eliminate the confounding effects of variable zooplankton lipid content on interpretation of $\delta^{13}C$ data, thereby increasing the accuracy of aquatic food web diagrams and dietary mixing models. Since the mass balance correction model also accurately predicts depletions in $\delta^{13}C$ values of fish tissues caused by variable fatty acid storage (Sweeting et al. 2006), we advocate more consistent use of this model to reduce bias in comparisons of zooplankton and fish $\delta^{13}C$ values and to provide a standard way of comparing results from other studies. The strategies for lipid correction developed in this investigation should also be applicable in other food web studies that involve comparisons of organisms with variable fatty acid contents such as those in polar marine regions or soil-dwelling ecosystems (Ponsard and Arditi 2000; Graeve et al. 2001).

Fatty acid content and zooplankton $\delta^{13}C_{bulk}$ values—The stable carbon isotope compositions of zooplankton were up to 5‰ depleted in ^{13}C because of the influence of fatty acid storage. The difference ($\Delta^{13}C_{ex\text{-}non}$) between nonextracted ($\delta^{13}C_{bulk}$) and lipid-extracted ($\delta^{13}C_{ex}$) values of zooplankton steadily increased with fatty acid content because fatty acids are depleted in ^{13}C relative to other biochemical constituents such as proteins. This depletion predominantly stems from the enzymatic oxidation of pyruvate to the acetyl CoA precursors used to synthesize fatty acids (DeNiro and Epstein 1977). Values of $\Delta^{13}C_{ex\text{-}non}$ for zooplankton observed in the present study were much greater than have previously been reported (e.g., ~1.2‰ by Kling and Fry 1992; Kiljunen et al. 2006). Our highest calculated values of $\Delta^{13}C_{ex\text{-}non}$, up to 5.0‰ for some calanoid copepods, are similar to those measured for the lipid-rich liver tissue of sea bass (Sweeting et al. 2006). The ability of zooplankton to store large amounts of fatty acids, mainly in the form of triacylgylcerols for energy reserves (Vanderploeg et al. 1992; Arts et al. 1993), creates an opportunity for significant bias toward depleted $\delta^{13}C_{bulk}$ values. Therefore, the significant effects of fatty acid content on zooplankton $\delta^{13}C$ values are closely connected to lipid storage strategies of specific zooplankton groups.

Copepods contained more fatty acid reserves than cladocerans and therefore their $\delta^{13}C_{bulk}$ values were influenced to a greater extent. Large fatty acid reserves resulted in values of $\Delta^{13}C_{ex\text{-}non}$ that were 3.0‰ or greater for nearly 40% of all copepod samples. In contrast, more than 80% of cladocerans had $\Delta^{13}C_{ex\text{-}non}$ values that were less than 2.0‰ due to their low fatty acid content. Therefore, a substantial portion of the frequently observed differences in $\delta^{13}C_{bulk}$ values between coexisting cladocerans and copepods (Mathews and Mazumder 2003) may be explained by differences in fatty acid content. The rapid development and short, seasonal life cycle of many cladocerans may lead them to allocate more of their fatty acid energy reserves toward fast growth and reproduction rather than storage (Ventura and Catalan 2005; Smyntek 2006). Compared with the cladocerans, both calanoid and cyclopoid copepods spanned a broader range of fatty acid content, which accounts for the strong correlation with observed $\Delta^{13}C_{ex\text{-}non}$ values. For some copepods (*Limnocalanus sp., Diaptomus laticeps*, and *Cyclops strenuus abyssorum*) the ability to accumulate large quantities of fatty acids may permit survival in the deep, cold-water habitats that they frequent during at least part of their life cycle (Barbiero et al. 2000; Thackeray et al. 2005; Smyntek 2006). This ability may also allow these adult copepods to remain active over the winter since fatty acids are important in maintaining cell membrane fluidity in cold environments and are also a dense form of energy storage that can be slowly utilized during periods of low food availability (Farkas 1979; Tessier et al. 1983; Vanderploeg et al. 1992). The varying abilities of distinct types of zooplankton to accumulate large lipid reserves that affect their bulk $\delta^{13}C$ values underscore the need for a comparison of $\delta^{13}C_{ex}$ for a more accurate assessment of resource competition and predator–prey interactions within zooplankton communities.

Individual fatty acids in copepods were also frequently more depleted in ^{13}C than those of cladocerans. For example, copepods from Lake Michigan contained hexadecanoic acid that was depleted in ^{13}C by more than 2‰ relative to the same fatty acid present in *Daphnia* sp. (Smyntek unpubl. data). The average difference between the measured $\delta^{13}C_{ex}$ and the directly measured $\delta^{13}C_{fatty\ acid}$ values, denoted D, was significantly larger for copepods compared with cladocerans in both basins of Windermere and Grasmere. The $\delta^{13}C$ values of different fatty acids can vary by up to ~10‰ (Abrajano et al. 1994; Veefkind 2003), and copepods and cladocerans have distinct fatty acid

profiles (Farkas 1979; Smyntek 2006). Compared with cladocerans, copepods often contain a greater abundance of fatty acids that are the most depleted in ^{13}C such as α-linolenic acid and other polyunsaturated fatty acids (Veefkind 2003). This will result in lower $\delta^{13}C_{fatty\ acid}$ values and thus higher values of D for copepods. In addition, the lower-temperature habitats often utilized more frequently by copepods relative to cladocerans (Barbiero et al. 2000; Thackeray et al. 2005) may lead to newly biosynthesized lipids that are more depleted in ^{13}C because of increased enzymatic fractionation at lower temperatures (DeNiro and Epstein 1977). However, there may be other metabolic factors affecting the differences in isotopic composition of fatty acids or proteins in copepods and cladocerans, and detailed studies are still needed to better assess this phenomenon.

Relation between zooplankton and particulate organic matter (POM) $\delta^{13}C$ values—The influence of lipid storage by zooplankton may also help to explain reports of large depletions in their $\delta^{13}C$ values relative to those of the available POM across many aquatic ecosystems (del Giorgio and France 1996; Grey et al. 2000). Consumers are generally slightly enriched in ^{13}C by up to 1‰ relative to their diet. Therefore zooplankton would be expected to be enriched in ^{13}C relative to POM forming the base of the pelagic food web if they are utilizing this food source. However, many studies have demonstrated that zooplankton are frequently depleted in ^{13}C by 2–5‰ compared with POM (Grey et al. 2000; Mathews and Mazumder 2005). Previous explanations of this widespread phenomenon have focused on the potential for zooplankton grazing on allochthonous material (del Giorgio and France 1996; Grey et al. 2000), methanogenic bacterial production (Jones et al. 1999; Bastviken et al. 2003), or deep-dwelling algae that fix isotopically light respired carbon dioxide (France et al. 1997; Mathews and Mazumder 2003). However, daphnids, which are known to feed nonselectively on a wide range of phytoplankton taxa (DeMott 1986), also show depletions in ^{13}C relative to POM in lakes in which phytoplankton dominate the POM (Grey et al. 2000; Smyntek 2006). On the basis of the strong correlation between fatty acid content and $\Delta^{13}C_{ex\text{-}non}$ for copepods as well as cladocerans, the accumulation of large lipid reserves by zooplankton relative to phytoplankton appears to be at least partially responsible for the frequently observed depletion in zooplankton $\delta^{13}C$ values relative to POM. Zooplankton can store significantly greater amounts of fatty acid than the phytoplankton that compose POM (Persson and Vrede 2006), with observed differences as great as an order of magnitude (Smyntek 2006). This can hinder a simple comparison of $\delta^{13}C_{bulk}$ values of zooplankton with those of potential POM food sources. For example, seasonal changes in atomic C : N ratios of several zooplankton species were found to explain a greater amount of variation in zooplankton $\delta^{13}C_{bulk}$ values than seasonal changes in $\delta^{13}C$ values of POM (Mathews and Mazumder 2005). This suggests that temporal variation in zooplankton fatty acid content can affect interpretation of zooplankton $\delta^{13}C$ values. Thus the strong influence of large fatty acid reserves in some zooplankton, particularly copepods, may often bias $\delta^{13}C_{bulk}$ values and mask their true relation with phytoplankton food sources. Since proteins and carbohydrates compose a large portion of the organic carbon content of phytoplankton (Geider and La Roche 2002), and $\delta^{13}C$ values of carbohydrates in phytoplankton are generally similar (within 1–2‰) to $\delta^{13}C$ values of their proteins (Hayes 2001; Teece and Fogel 2007), a comparison of $\delta^{13}C$ values of POM with $\delta^{13}C_{ex}$ of zooplankton may be a reasonable approach to examining these potential relations at the base of an aquatic food web.

Comparison of zooplankton and fish $\delta^{13}C$ values—The depletions in $\delta^{13}C_{bulk}$ values due to high fatty acid content can greatly distort the importance of lipid-rich zooplankton to the diet of planktivorous fish. The contributions of zooplankton to the diet of planktivorous fish are frequently estimated by using static mixing models that involve measurements of the $\delta^{13}C$ values of fish as well as potential zooplankton prey items (Vander Zanden et al. 1999; Philips and Gregg 2001). These models calculate the proportions of prey items assimilated by the fish on the basis of the assumption that the $\delta^{13}C$ value of the fish will reflect the integrated $\delta^{13}C$ values of its diet plus any small enrichment associated with metabolic processing of dietary carbon. Dorsal white muscle tissue, which is typically low in fatty acid content, is considered a reliable tissue for stable isotope analysis of fish diet (Pinnegar and Polunin 1999). The large values of $\Delta^{13}C_{ex\text{-}non}$ for zooplankton, particularly copepods, are up to five times greater than the average values of $\Delta^{13}C_{ex\text{-}non}$ typically observed for fish white muscle tissue (Sweeting et al. 2006; Murry et al. 2006). Therefore, a failure to correct $\delta^{13}C_{bulk}$ values of zooplankton for lipid content can severely compromise the accuracy of dietary mixing models for planktivorous fish by greatly underestimating or even excluding proportions of lipid-rich zooplankton, such as copepods, from fish diet. For example, in a recent survey of the diet of alewife (*Alosa pseudoharengus*), a common planktivorous fish in several of the North American Great Lakes, stable carbon isotope results agreed with gut content analyses only after corrections were made for zooplankton lipid content (Storch 2005). Future studies of fish diet using stable carbon isotope analysis of zooplankton and fish muscle tissue must address differences in lipid content between these two distinct types of samples. This can be achieved by extracting lipids from samples before analysis or by using mathematical correction models to determine $\delta^{13}C_{ex}$ of zooplankton and fish samples.

Evaluation of mathematical models for correcting zooplankton $\delta^{13}C$ values—The present data encompassing a range of lakes and different zooplankton species indicate that the mass balance model provides a more accurate correction of the $\delta^{13}C_{bulk}$ values of zooplankton for variable fatty acid content than the lipid normalization model. The lipid normalization model consistently underestimated the influence of fatty acid storage on $\delta^{13}C_{bulk}$ values in zooplankton, particularly for copepods. This resulted in predicted values of $\delta^{13}C_{ex}$ that were significantly

lower than measured values. Leggett (1998) reported a similar underestimation of the effects of lipid extraction on $\delta^{13}C$ values when applying the lipid normalization model in his study of opossum shrimp (*Mysis reticula*) in Lake Ontario. Since this model is based on theoretical considerations and empirical data derived from marine fish and crustaceans (McConnaughey 1978), it may be inadequate for freshwater organisms.

In contrast, the mass balance correction model provides a simple and efficient method to accurately predict $\delta^{13}C_{ex}$ for both copepods and cladocerans using atomic C : N ratios of the nonextracted samples and the average values of D (6.3‰ ± 1.3‰) and $C:N_{ex}$ (4.2 ± 0.4) as shown in Eq. 5.

$$\delta^{13}C_{ex} = \delta^{13}C_{bulk} + 6.3\left(\frac{C:N_{bulk} - 4.2}{C:N_{bulk}}\right) \quad (5)$$

The low error and strong statistical relation ($R^2 = 0.95$) for the model-predicted values of $\delta^{13}C_{ex}$ compared with the observed values across a range of zooplankton in a variety of lake systems attest to the robust assumptions of this model. The potential sources of error in using Eq. 5 for correcting zooplankton $\delta^{13}C$ values for fatty acid content are the accuracies of the constants representing D (6.3‰) and $C:N_{ex}$ (4.2). Although variation in the value of D can affect the predicted value of $\delta^{13}C_{ex}$, the greatest effect is confined to samples with high atomic $C:N_{bulk}$ ratios (>7.0), which composed only 20% of all samples. For the other zooplankton samples ($C:N_{bulk} < 7.0$), deviations in the average value of D (6.3‰) by up to 1.5‰, a range that included more than 85% of the measured D values (Table 2), affect the predicted value of $\delta^{13}C_{ex}$ by less than 0.6‰. The low coefficient of variation for $C:N_{ex}$ (9.1%) for over 10 species of zooplankton from nine diverse lake systems suggests that the average of 4.2 is representative of most zooplankton. This is supported by other work that has found similar values of $C:N_{ex}$ for fish muscle (Sweeting et al. 2006), brown shrimp (Fry et al. 2003), and opossum shrimp (Leggette 1998). In addition, deviation in $C:N_{ex}$ by ±0.5 from the average value of 4.2, which encompassed more than 90% of the measured values, will affect the predicted value of $\delta^{13}C_{ex}$ by a maximum of 0.6‰ for zooplankton samples with atomic $C:N_{bulk} \geq 5.0$. Thus the potential for error due to the accuracy of these two constants is low (0.6‰) relative to the large effects of fatty acid storage on zooplankton $\delta^{13}C_{bulk}$ values (up to 5.0‰). Therefore Eq. 5 provides a robust calculation of $\delta^{13}C_{ex}$, particularly for samples that have atomic $C:N_{bulk}$ values of 5.0–7.0. Finally, since average values of D and $C:N_{ex}$ are derived from measurements of both copepods and cladocerans, this equation should provide an accurate calculation of $\delta^{13}C_{ex}$ for mixed pelagic zooplankton samples as well.

We were unable to determine whether allowing zooplankton to evacuate their gut contents had a direct effect on their stable isotope composition or the value of $\Delta^{13}C_{ex\text{-}non}$, as paired samples were not analyzed. However, the absolute deviation from the model-predicted values of $\delta^{13}C_{ex}$ relative to the measured values was not significantly different (two-sample t-test, $p = 0.840$) from zooplankton collected from the Lake District, which were allowed to evacuate their guts, and those from the Great Lakes, where no gut evacuation was allowed. As the model was able to accurately predict the values of $\delta^{13}C_{ex}$ for zooplankton that had undergone each sample preparation technique, we would suggest that allowing zooplankton to evacuate their guts before analysis had little effect on the direct measurement, and also the predicted, stable isotope composition of zooplankton from our study lakes. Similarly, Feuchtmayr and Grey (2003) observed no significant difference between the $\delta^{13}C$ values of paired samples of zooplankton from a mesotrophic lake, either allowing for gut evacuation or not. From our results we are unable to conclusively address the issue of gut evacuation before stable isotope analysis.

The accuracy of mass balance correction models, which has previously been confirmed for fish muscle tissue (Sweeting et al. 2006) and is now verified for freshwater zooplankton, attests to their usefulness in providing a better means to assess the diet of planktivorous fish and predatory zooplankton. Most stable carbon and nitrogen isotope analysis is conducted with an elemental analyzer coupled to an IRMS in a continuous flow setup, which allows atomic C : N ratios of the samples to be measured simultaneously with $\delta^{13}C$ and $\delta^{15}N$ values. Thus the raw data tools necessary for mass balance correction are already available with little extra analysis needed. We recommend the use of the mass balance correction in Eq. 5 for the analysis of most zooplankton samples, particularly those with atomic $C:N_{bulk}$ of 5.0–7.0. If extra caution is needed for samples with high atomic $C:N_{bulk}$ ratios (>7.0), the potential for error can be greatly reduced by directly measuring $\delta^{13}C_{ex}$ and $\delta^{13}C_{fatty\ acid}$ for a subset of samples to derive a value of D specific to the zooplankton of interest. Incidentally, the atomic C : N of the extracted sample ($C:N_{ex}$) can be measured simultaneously with $\delta^{13}C_{ex}$, which further constrains potential error for the mass balance model. In addition to providing accurate, less biased $\delta^{13}C$ values, mass balance correction models greatly reduce expense, time of analysis, and the amount of sample material needed for analysis. Therefore we strongly recommend the use of mass balance correction models to derive values of $\delta^{13}C_{ex}$ for zooplankton that can be used in dietary mixing models and other aspects of food web analysis.

The large and variable lipid or fatty acid content of zooplankton can greatly influence their bulk $\delta^{13}C$ values. When bulk $\delta^{13}C$ values of zooplankton are compared with samples with lower lipid content, such as phytoplankton or fish muscle tissue, there is potential for altered interpretations of stable isotope food web diagrams and significantly biased results of dietary mixing models. Mass balance correction models using the atomic C : N of a sample as an indicator of lipid content can accurately predict values of the lipid-extracted sample, $\delta^{13}C_{ex}$, for a range of zooplankton species. Future studies using stable carbon isotope analysis of zooplankton and fish muscle tissue should have mass balance correction models as an efficient and effective method to constrain bias caused by variation in lipid content among samples.

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