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METHODOLOGICAL INSIGHTS

A revised model for lipid-normalizing $\delta^{13}C$ values from aquatic organisms, with implications for isotope mixing models

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Summary

1. Stable isotope analyses coupled with mixing models are being used increasingly to evaluate ecological management issues and questions. Such applications of stable isotope analyses often require simultaneous carbon and nitrogen analyses from the same sample. Correction of the carbon isotope values to take account of the varying content of ^{13}C-depleted lipids is then frequently achieved by a lipid-normalization procedure using a model describing the relationship between change in $\delta^{13}C$ following lipid removal and the original C:N ratio of a sample.

2. We evaluated the applicability of two widely used normalization models using empirical data for muscle tissue from a wide range of fish and for aquatic invertebrates. Neither normalization model proved satisfactory, and we present some modifications that greatly improve the fit of one of the models to the fish muscle data. For invertebrates we found no clear relationship between change in $\delta^{13}C$ following lipid removal and the original C:N ratio.

3. We also examined the effect of lipid-normalization on the output of a mixing model designed to calculate the proportional contribution of prey items to the diet of a consumer. Mixing model output was greatly influenced by whether prey or consumer values alone or together were lipid-normalized and we urge caution in the interpretation of results from these models pending further experimental evidence.

4. *Synthesis and applications*. We describe a revised lipid-normalization model that should be applicable to a wide range of marine and freshwater fish species in studies applying stable isotope analyses to ecological management issues. However, we strongly advise against applying these kinds of lipid-normalization models to aquatic invertebrate data. The interpretation of outputs from mixing models is greatly influenced by whether the carbon isotope data have been lipid-normalized or not.

Key-words: $\delta^{13}C$, aquatic invertebrates, C:N ratio, fish, lipids, mixing models, validation

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Introduction

Stable isotope analysis (SIA) of carbon is now widely used to quantify food sources of aquatic consumers. Following experiments by DeNiro & Epstein (1978) on the influence of diet on the distribution of carbon isotopes in animals, it is widely assumed that the $\delta^{13}C$ value of an organism is within 1‰ of that of its diet. However, it is known that there is considerable natural variation in isotope ratios associated with the proportion and allocation of dietary constituents such as lipids, protein and carbohydrates (Gannes, O'Brien & Martínez del Rio 1997; Focken & Becker 1998;

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Gannes, Martínez del Rio & Koch 1998; Sotiropoulos, Tonn & Wassenaar 2004). In particular, lipids are known to be ^{13}C-depleted (have lower δ^{13}C) relative to other major tissue constituents (DeNiro & Epstein 1978; Focken & Becker 1998; Thompson *et al*. 2000). This variation in δ^{13}C caused by lipid composition potentially complicates interpretation of dietary sources of carbon (Rolff & Elmgren 2000). A difference in lipid composition can give rise to variation in δ^{13}C values between individuals higher than the commonly assumed 1‰ difference between trophic levels, and hence may lead to biased interpretation of isotope results (Focken & Becker 1998). Because this problem has long been recognized in aquatic stable isotope studies (McConnaughey & McRoy 1979; Peterson & Fry 1987), lipid-extraction methods, using chloroform-methanol or hexane-isopropanol solvent mixtures, prior to analysis of δ^{13}C have frequently been applied. It has been argued that such 'lipid-corrected' δ^{13}C values more directly reflect assimilated carbon, whereas uncorrected δ^{13}C values reflect the combined effects of assimilation and lipid synthesis (Power, Guiguer & Barton 2003).

Modern continuous flow isotope ratio mass spectrometry (CF-IRMS) can conveniently measure both carbon and nitrogen isotopes from combustion of a single sample and many studies make use of both isotope values. However, lipid-extraction procedures have been shown to alter δ^{15}N values (Sotiropoulos, Tonn & Wassenaar 2004) and therefore it is recommended to analyse separate samples for carbon and nitrogen isotopes, extracting lipids only from the carbon sample. As this greatly increases the time and costs of analyses, several lipid-normalization methods calculated from C:N ratios have been developed for δ^{13}C values. Lipid content of tissue can be estimated quite accurately from its C:N ratio (McConnaughey & McRoy 1979; Schmidt *et al*. 2003) and most CF-IRMS systems provide mass percentages of carbon and nitrogen from which the C:N ratio can readily be derived. Rolff & Elmgren (2000) used regression of δ^{13}C on the C:N ratio to correct plankton samples for lipids and avoided the effect of location by setting an independent intercept for each location. Alexander *et al*. (1996) presented a simple method in which fat-free δ^{13}C values are estimated by simply adding 6‰ multiplied by the percentage fat content to the measured δ^{13}C values, assuming 6‰ to be the approximate difference between protein and lipid δ^{13}C values (McConnaughey & McRoy 1979). However, the most widely applied method in aquatic science has been that of McConnaughey (1978), later published in McConnaughey & McRoy (1979). One equation estimates the lipid content of the sample, which is then used in a second equation to calculate lipid-normalized δ^{13}C values. However, the first equation was based on rather limited C:N and lipid content data from Pacific fish and crustaceans. Kline (1997) has shown indirectly that the model removes the effect of lipids but it has never been validated properly. An extensive literature search revealed only one paper in which the method was tested before use (Satterfield & Finney 2002). This is surprising, as some authors have presented quite large differences between lipid-normalized and original values (Kline 1999), and some have used the method to correct values for freshwater organisms (Power *et al*. 2002) even though the model was developed using data from marine environments.

We first evaluated the application of the two most widely used lipid-normalization methods to fish and aquatic invertebrates, and modifications that improved their applicability while highlighting limitations in their use. We then used data from a seal species and its prey to illustrate how lipid-normalization can affect the outputs from mixing models used to determine the diet composition of a consumer.

Materials and methods

DATA COLLECTION, LIPID-EXTRACTION AND STABLE ISOTOPE ANALYSIS

Three sets of fish and invertebrate data were used for evaluation and development of the models. The data set of freshwater, brackish water and marine organisms used for modelling was collected in 2002–04 and included 12 species of fish and five groups of invertebrates (Table 1). An independent data set (Table 2) was used for model validation and included 10 fish species caught in 2002 and 2004 from the Bothnian Bay (northern Baltic Sea). The third data set included 20 wild-caught Baltic salmon *Salmo salar* L., 15 herring *Clupea harengus membras* L. and nine sprat *Sprattus sprattus* L. samples collected in 2003 around the Baltic Sea. These data were used to evaluate the relationship between C:N and lipid content used in lipid-normalization models.

In the first and second data sets, a small sample of the dorsal muscle was taken from each fish and either freeze-dried (samples from Finland) or oven-dried (all other samples) at 60 °C to constant weight. Invertebrates were oven-dried whole at 60 °C to constant weight. Tests (J. Grey, unpublished data) had shown no effect of drying method on isotope values. Dried samples were ground to a fine powder using either a freezer mill (6750 SPEX; CertiPrep Inc., Metuchen, NJ) or mortar and pestle. Each sample was divided in two and one part was treated to remove lipids according to the protocol of Blight & Dyer (1959). Samples were placed in glass vials and stored frozen prior to isotope analysis. Stable isotope analyses were made by CF-IRMS. Samples collected from Ireland and Germany were analysed using a Eurovector elemental analyser connected to a Micromass Isoprime CF-IRMS at the Max Planck Institute for Limnology in Plön, Germany. Samples collected from the Baltic Sea were analysed in a similar manner at the University of Jyväskylä, Finland, using a Carlo Erba Flash EA1112 elemental analyser connected to a Thermo Finnigan DELTA^{plus}Advantage CF-IRMS. Results are expressed using the standard δ notation as parts per thousand (‰) difference from the

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Table 1. Fish and invertebrate samples from three different locations and habitats (FW, freshwater; BW, brackish water; M, marine). Number of measured samples (*n*), mean (± SD) $\delta^{13}C$ values and C:N ratios of untreated and treated (chemically lipid-extracted) samples, together with differences between treated and untreated $\delta^{13}C$ values ($\delta^{13}C_{treated}$–$\delta^{13}C$). All organisms were collected from the field, except for the carp which had been maintained in tanks for several years and fed on chironomid larvae and zooplankton. These data were used for model evaluation and development

				Untreated		Treated (lipid-extracted)		
Species or groups analysed	Habitat	Location	*n*	$\delta^{13}C$	C:N	$\delta^{13}C_{treated}$	C:N	$\delta^{13}C_{treated}$–$\delta^{13}C$
Fish								
Eel *Anguilla anguilla* (L.)*	FW	Ireland	67	–27·12 ± 3·77	9·36 ± 9·32	–23·68 ± 2·54	3·08 ± 0·19	3·44 ± 2·39
Carp *Cyprinus caprio* L.	FW	Germany	14	–16·66 ± 0·86	4·02 ± 0·38	–15·70 ± 0·85	3·02 ± 0·26	0·96 ± 0·41
Eel *Anguilla anguilla* (L.)	BW	Ireland	27	–23·92 ± 1·76	3·76 ± 0·68	–22·60 ± 1·32	3·18 ± 0·10	1·31 ± 0·99
Salmon *Salmo salar* L.	BW	Baltic Sea	10	–22·72 ± 1·50	6·78 ± 2·63	–20·04 ± 0·80	3·46 ± 0·40	2·68 ± 1·12
Baltic herring *Clupea harengus membras* L.	BW	Baltic Sea	10	–22·53 ± 1·47	3·66 ± 0·33	–21·41 ± 1·78	3·19 ± 0·06	1·11 ± 0·47
Eel *Anguilla anguilla* (L.)	M	Ireland	34	–17·34 ± 1·41	3·83 ± 1·36	–16·27 ± 0·92	3·27 ± 0·06	1·07 ± 0·96
Lesser sand eel *Ammodytes tobianus* L.	M	Ireland	30	–18·12 ± 0·21	3·20 ± 0·13	–17·46 ± 0·27	2·91 ± 0·06	0·67 ± 0·37
Flounder *Platichthys flesus* (L.)	M	Ireland	15	–16·50 ± 1·31	3·00 ± 0·12	–16·13 ± 1·24	2·88 ± 0·06	0·37 ± 0·22
Pollock *Pollachius pollachius* (L.)	M	Ireland	10	–16·43 ± 0·87	3·00 ± 0·06	–15·89 ± 0·82	2·79 ± 0·05	0·53 ± 0·33
Black goby *Gobius niger* L.	M	Ireland	3	–16·33 ± 0·91	2·95 ± 0·02	–16·23 ± 0·85	2·85 ± 0·03	0·10 ± 0·09
Corkwing wrasse *Symphodus melops* (L.)	M	Ireland	4	–16·36 ± 0·44	3·18 ± 0·14	–16·62 ± 0·42	2·86 ± 0·03	0·74 ± 0·34
Trout *Salmo trutta* L.	M	Ireland	1	–16·78	2·99	–16·73	2·95	0·05
Thick-lipped grey mullet *Chelon labrosus* (Risso)	M	Ireland	1	–16·79	3·47	–16·65	3·02	0·14
Cod *Gadus morhua* L.	M	Ireland	1	–14·60	2·94	–14·48	2·91	0·12
Invertebrates								
Chironomidae larvae	FW	Germany	24	–40·95 ± 11·60	6·74 ± 2·02	–39·74 ± 11·33	–	1·20 ± 0·67
Bulk zooplankton	FW	Germany	15	–34·96 ± 7·27	5·64 ± 0·56	–32·19 ± 5·64	4·07 ± 0·27	2·77 ± 2·37
Limnocalanus sp.	BW	Baltic Sea	3	–26·76 ± 0·29	8·15 ± 0·63	–25·11 ± 0·23	3·44 ± 0·09	1·66 ± 0·06
Monoporeia affinis Lindström	BW	Baltic Sea	2	–24·58 ± 0·06	7·94 ± 0·13	–22·74 ± 0·30	4·12 ± 0·42	1·84 ± 0·24
Saduria entomon (L.)	BW	Baltic Sea	3	–20·79 ± 2·26	5·69 ± 0·24	–19·65 ± 3·02	4·83 ± 0·04	1·14 ± 0·85

*Yellow-phase freshwater eels.

Table 2. Fish samples from the brackish water Bothnian Bay (northern Baltic Sea). Number of samples (*n*), mean (± SD) $\delta^{13}C$ values and C:N ratios of untreated and treated (chemically lipid-extracted) fish muscle, together with difference between treated and untreated $\delta^{13}C$-values ($\delta^{13}C_{treated}$–$\delta^{13}C$) and their standard deviation. These data were used for model validation

		Untreated		Treated (lipid-extracted)		
Species analysed	*n*	$\delta^{13}C$	C:N	$\delta^{13}C_{treated}$	C:N	$\delta^{13}C_{treated}$–$\delta^{13}C$
Burbot *Lota lota* (L.)	5	–22·76 ± 1·91	3·29 ± 0·04	–22·42 ± 1·82	3·19 ± 0·08	0·34 ± 0·41
Whitefish *Coregonus lavaretus* (L.)	15	–22·13 ± 0·81	3·25 ± 0·14	–21·86 ± 0·79	3·14 ± 0·08	0·26 ± 0·09
Pike *Esox lucius* L.	2	–20·67 ± 0·22	3·22 ± 0·13	–20·53 ± 0·32	3·13 ± 0·16	0·13 ± 0·10
Sculpin *Myoxocephalus quadricornis* (L.)	1	–21·36	3·34	–20·66	3·10	0·70
Smelt *Osmerus eprlanus* (L.)	7	–22·51 ± 0·57	3·28 ± 0·05	–21·78 ± 0·51	3·18 ± 0·04	0·73 ± 0·20
Ruffe *Gymnocephalus cernuus* (L.)	5	–21·51 ± 0·61	3·34 ± 0·01	–21·11 ± 0·68	3·21 ± 0·06	0·41 ± 0·10
Perch *Perca fluviatilis* L.	3	–22·16 ± 085	3·28 ± 0·03	–22·1 ± 0·79	3·07 ± 0·05	0·06 ± 0·06
Salmon *Salmo salar* L.	10	–22·48 ± 0·99	5·59 ± 1·15	–19·8 ± 0·49	3·37 ± 0·21	2·67 ± 0·74
Vendace *Coregonus albula* (L.)	5	–23·36 ± 0·33	3·20 ± 0·04	–23·02 ± 0·40	3·08 ± 0·06	0·34 ± 0·09
Baltic herring *Clupea harengus membras* L.	9	–23·34 ± 0·0.93	3·39 ± 0·19	–22·76 ± 0·93	3·16 ± 0·05	0·58 ± 0·22

international standard. The reference materials used were internal standards of known relation to the international standards of Vienna Pee Dee belemnite (for carbon) and atmospheric N_2 (for nitrogen). Precision was better than 0·2‰, based on the standard deviation of replicates of the internal standards. Sample analysis also yielded percentage carbon and nitrogen from which C:N ratios (by weight) were derived.

To evaluate the relationship between lipid content and C:N ratio, whole fish homogenates from the third data set were freeze-dried and Soxhlet-extracted for 20 h with toluene. The lipid content was determined gravimetrically after evaporation of the toluene (Kiviranta *et al*. 2003). Although toluene is not commonly used as a solvent for lipid-extraction, the Finnish National Public Health Institute laboratory has regularly participated in international intercalibration studies and the lipid values were in exact agreement with calibration standards.

EVALUATION OF THE MODELS AND MODEL MODIFICATIONS

Two lipid-normalization models were evaluated by comparing model predictions with observed data from samples with and without chemical lipid-extraction. The lipid-normalization model of McConnaughey & McRoy (1979) is based on two equations:

$$L = \frac{93}{1 + (0{\cdot}246 \times (C:N) - 0{\cdot}775)^{-1}} \qquad \text{eqn 1}$$

$$\delta^{13}C' = \delta^{13}C + D \times \left(I + \frac{3{\cdot}90}{1 + 287/L} \right) \qquad \text{eqn 2}$$

where L is the proportional lipid content of the sample and $\delta^{13}C'$ is the lipid-normalized value of the sample; C and N are the proportions of carbon and nitrogen in the sample; $\delta^{13}C$ is the measured value of the sample; D is the isotopic difference between protein and lipid; and I is a constant (assigned a value of –0·207). McConnaughey & McRoy (1979) assigned a value to D of 6‰ based on literature data. In their model, a C:N ratio of 4 was assumed to be normal for animal tissue, so animals with C:N > 4 have less negative $\delta^{13}C'$ values and animals with C:N < 4 have more negative $\delta^{13}C'$ values compared with measured $\delta^{13}C$ values.

In the Alexander *et al*. (1996) model, lipid-normalized δ-values are simply estimated as:

$$\delta^{13}C' = \delta^{13}C + D \times \frac{L}{100} \qquad \text{eqn 3}$$

in which all parameters are the same as described for equation 2. Alexander *et al*. (1996) used chemically analysed lipid concentrations (L) but we estimated lipid content using equation 1. Alexander *et al*. (1996) normalized $\delta^{13}C$ values to zero lipid content and not to a constant as used by McConnaughey & McRoy (1979). For this reason, $\delta^{13}C'$ values generated by Alexander *et al*. (1996) model are always less negative than the original ($\delta^{13}C$) values.

For model evaluation, differences were calculated between chemically lipid-extracted ($\delta^{13}C_{treated}$) and original ($\delta^{13}C$) values and between modelled ($\delta^{13}C'$) and original ($\delta^{13}C$) values. Differences were then plotted against observed C:N ratios for fish and invertebrates separately. As neither model provided a satisfactory fit to our observed data, we modified the McConnaughey & McRoy (1979) model (which has a strong theoretical background based on biological rationale; McConnaughey 1978). As there was no clear pattern between C:N ratio and $\delta^{13}C_{treated}$–$\delta^{13}C$ values in our invertebrate data, we developed the modified model for fish only. In our model modification, the two constants in equation 2 were re-estimated. The first of these, D, defines the slope curvature of the model, while the second constant, I, defines the intersection on the x-axis (itself allocated a value of 4 in the model of McConnaughey & McRoy 1979). These two parameters were iteratively re-estimated by fitting the original equation to our observed fish data using an SPSS statistical software package (SPSS 12·0·1, SPSS Inc., Chicago, IL). Re-estimation used the Levenberg–Marquard non-linear

optimization method and a loss function of sum of squared residuals. Starting values for estimated parameters were the same as they are in the original model of McConnaughey & McRoy (1979). Estimation was continued until the convergence criterion ($1{\cdot}0^{E-8}$) was reached.

VALIDATION OF THE MODELS

The modified model was validated using the independent fish data (Table 2). For each fish, lipid-normalized values were estimated using the modified model. For comparison, estimates were also calculated using the two previous models. The different estimates given by these three models ($\delta^{13}C'$) were compared visually by inspection of observed vs. predicted plots and also as the calculated modelling efficiency (EF; Mayer & Butler 1993) defined as follows:

$$EF = 1 - \frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y}_i)^2} \qquad \text{eqn 4}$$

where y_i is the observed and $\hat{y}_i$ the predicted value. Modelling efficiency gives an indication of goodness-of-fit, with a value of 1 describing a perfect fit and values approaching 0 indicating poor model performance. Negative values of EF indicate that the average value of all measured values is a better predictor than the model used. (Mayer & Butler 1993).

MIXING MODELS

Carbon isotopes are often used in mixing models with other isotope values to calculate proportions of different food sources in the diet of a consumer. We investigated how lipid-normalization could influence such calculations of diet contributions to Baltic ringed seal *Phoca hispida botnica* (Gmelin) using four alternative scenarios covering all possible combinations of lipid correction of predator and prey $\delta^{13}C$ values. In the first, $\delta^{13}C$ values of both predator and prey species remained untreated. In the second scenario $\delta^{13}C$ values of both predator and prey species were normalized for lipids. In the third scenario only $\delta^{13}C$ values of prey species were lipid-normalized. In the fourth scenario only $\delta^{13}C$ values of predator were lipid-normalized. According to dietary studies that rely on identification of the remains of hard parts in scats and stomach or intestine contents, seals consume a variety of the prey species in the Baltic Sea (Söderberg 1975; Tormosov & Rezvov 1978). For the mixing model scenarios we analysed average $\delta^{13}C$ and $\delta^{15}N$ values from muscle samples of the five preferred prey species mentioned in the literature: herring, salmon, smelt, eelpout *Zoarces viviparous* (L.) and the isopod *Saduria entomon* (L.), as well as muscle from nine Baltic ringed seals (T. Sinisalo, unpublished data). Stable isotope values for prey species and seals were derived as described previously. Species-specific, lipid-normalized $\delta^{13}C$ values for the second, third and fourth scenarios were derived using the modified model presented in this study, except in the case of *S. entomon* and ringed seal, for which chemically lipid-extracted ($\delta^{13}C_{treated}$) values were used (Table 1) because the lipid-normalization method was not validated for these species. $\delta^{15}N$ values were not modified in any way.

We used the IsoSource computer program (Phillips & Gregg 2003) to determine the contribution of each prey species to the diet of the seals. IsoSource calculates all possible combinations of each dietary source contribution (0–100%) in small increments. In all three scenarios the model tolerance was set to ± 0·25% and the contribution of each source was calculated in 1% increments. Prior to modelling, prey $\delta^{15}N$ and $\delta^{13}C$ values were corrected for trophic enrichment using respective fractionation factors of 2·4‰ and 1·3‰, appropriate for seals according to Hobson *et al*. (1996). The results are reported as distributions of all feasible solutions, as recommended by Phillips & Gregg (2003), with 1–99th percentiles of the distribution ranges. Although we used real data for the IsoSource mixing model, the outputs are intended to be an illustrative example of how lipid-normalization may affect interpretation of the diets, rather than a robust description of the contributions each prey makes to the diet of Baltic ringed seals, as not all important dietary sources have necessarily been included and trophic fractionation factors between prey items and seal muscle are unverified for the four different scenarios.

Results

Fish $\delta^{13}C$ values of the 14 fish species varied between species and habitats (Table 1). Intra-species variation was high for some species. Considerable variation was also seen in the C:N ratios of untreated fish tissues, with highest values in eel, salmon and herring, which are generally considered to be lipid-rich fish (Murray & Burt 1969; Henderson & Tocher 1987). Some eels from freshwater habitats had exceptionally high C:N values; the maximum C:N measured from an individual eel was 63. Fish $\delta^{13}C$ values were higher after chemical lipid-extraction, with the biggest difference between untreated and lipid-extracted values seen in eel, salmon and herring tissues. After lipid-extraction, the C:N ratio of all fish species dropped close to a value of 3, with variation becoming lower (Table 1); the average post-extraction C:N ratio of all fish species was 3·08 (SD = 0·22).

There was a strong linear relationship between observed lipid content and lipid content estimated using equation 1 ($r^2 = 0{\cdot}970$, $P < 0{\cdot}001$, $n = 44$; Fig. 1). The model slightly overestimated the lipid content, with model residuals being on average 3·47%. However, modelling error was constant and the slope of the relationship between observed and predicted lipid content did not differ from unity (d.f. = 43, $t = 1{\cdot}269$, $P = 0{\cdot}211$). Modelling efficiency was also high (EF = 0·849). Hence model estimates could be considered rather accurate.

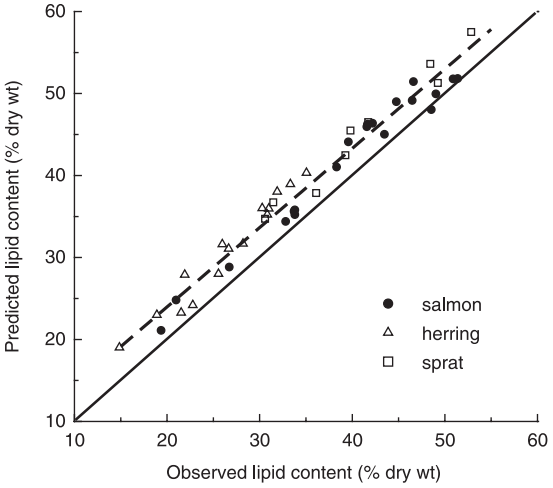

Fig. 1. Observed (chemically determined) vs. predicted (calculated using equation 1) lipid content of three Baltic Sea fish species used for evaluation of the McConnaughey & McRoy (1979) equation for estimating lipid content (L; equation 1). The solid line shows the 1:1 ratio for observed and predicted values and the dashed line is the fitted linear regression (see text for further details).

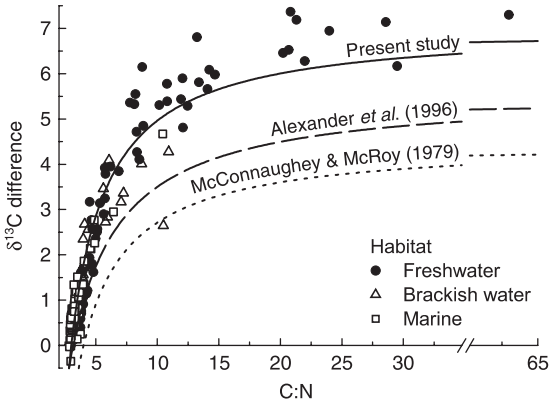

Fig. 2. Relationship between the $\delta^{13}C$ difference between lipid-extracted ($\delta^{13}C_{treated}$) and untreated ($\delta^{13}C$) values and the C:N ratios from untreated samples for the fish from three different habitats (Table 1). The solid line shows the $\delta^{13}C$ difference ($\delta^{13}C'$–$\delta^{13}C$) given by the modified lipid-normalization model derived from the data and, for comparison, dashed and dotted lines represent normalization curves calculated using the model of Alexander *et al.* (1996) and the original model of McConnaughey & McRoy (1979).

For fish there was a clear non-linear relationship between the difference between $\delta^{13}C$ of lipid-extracted and untreated samples ($\delta^{13}C_{treated}$–$\delta^{13}C$) and the C:N ratio of untreated samples. Comparison of the curves generated using the McConnaughey & McRoy (1979) and Alexander *et al.* (1996) models with the observed relationship showed that neither model provided a good fit to the data (Fig. 2). Both models markedly underestimated the difference between lipid-corrected $\delta^{13}C$ and untreated $\delta^{13}C$ values, particularly for samples with high C:N ratios. By iteratively fitting the McConnaughey & McRoy (1979) model to the observed

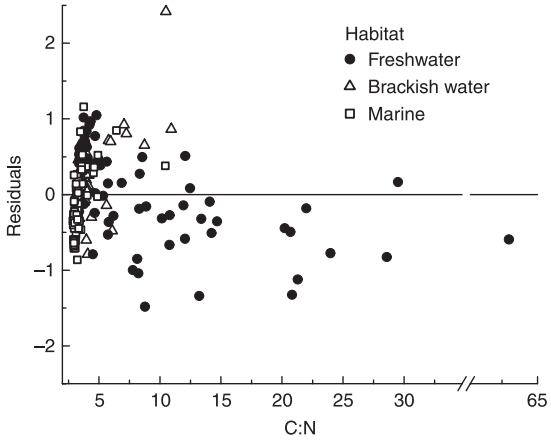

Fig. 3. Residuals from the fit of the modified lipid-normalization model to the observed fish data ($\delta^{13}C'$–$\delta^{13}C_{treated}$) in relation to untreated C:N ratio. The zero line represents a perfect fit of the model to the observations.

data set, changing only the two constants from the original equation (equation 2), the following revised equation was obtained:

$$\delta^{13}C' = \delta^{13}C + D \times \left(I + \frac{3{\cdot}90}{1 + 287/L} \right) \qquad \text{eqn 5}$$

in which the two re-estimated parameters were (± 95% confidence intervals) $D = 7{\cdot}018 \pm 0{\cdot}263$ and $I = 0{\cdot}048 \pm 0{\cdot}013$. The curve intersected the x-axis at 3·003, so according to this modified model fish muscle contained zero extractable lipid at a C:N ratio of approximately 3 (Fig. 2). In the data set one individual eel had an exceptionally high C:N ratio of 63, but the effect of removing this individual from the model was negligible and hence it could not be considered an outlier. Of the 227 samples, three showed very slightly negative values for the difference between lipid-extracted and untreated values ($\delta^{13}C_{treated}$–$\delta^{13}C$); this deviation below the theoretical value of zero for lipid-free tissue was within the analytical precision. The modified model had a high coefficient of determination ($r^2 = 0{\cdot}925$, $n = 227$) and the model residuals were rather evenly distributed, especially with C:N ratios < 20 (Fig. 3).

INVERTEBRATES

Like the fish, untreated freshwater invertebrates were also more ^{13}C-depleted than those from brackish waters (Table 1) and values varied more in the freshwater habitat. C:N ratios of untreated samples were rather high and after lipid-extraction C:N ratios did not drop to the same level as fish tissue, with the average C:N ratio after lipid-extraction being 4·03. The difference between untreated and lipid-extracted $\delta^{13}C$ values varied from 1·14 to 2·77. Inter-specific variation was highest in the zooplankton samples. All the lipid-normalization models used for fish were then compared using the invertebrate data. No clear relationship between

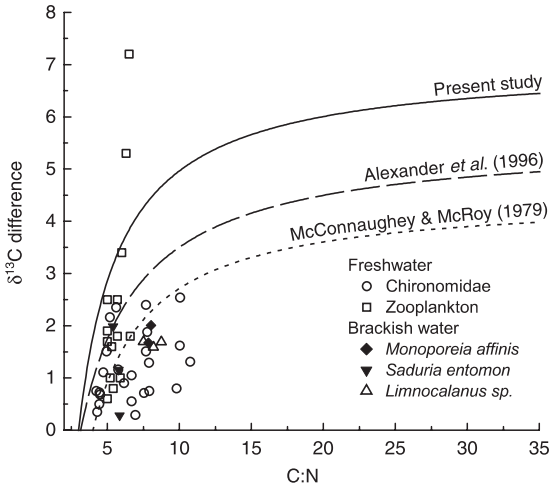

Fig. 4. Relationship between the $\delta^{13}C$ difference between lipid-extracted ($\delta^{13}C_{treated}$) and untreated ($\delta^{13}C$) values and the C:N ratios from untreated samples for the invertebrates (Table 1). Curves show the $\delta^{13}C$ difference ($\delta^{13}C'$–$\delta^{13}C$) calculated from the C:N ratio using the modified model of the present study (solid line), the model of Alexander *et al*. (1996) (dashed line) and the original model of McConnaughey & McRoy (1979) (dotted line).

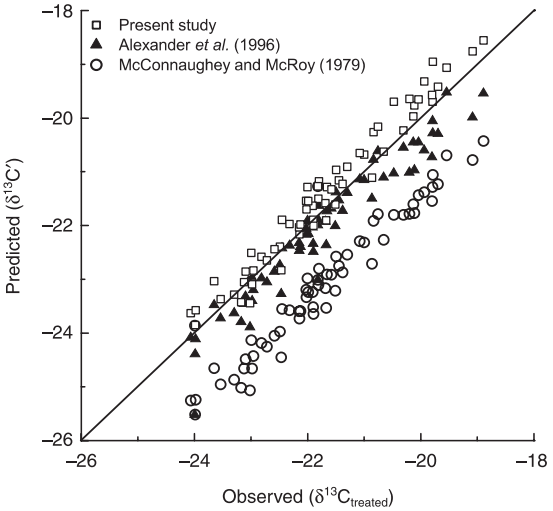

Fig. 5. Observed (chemically lipid-extracted) and predicted (from the three different lipid-normalization models) $\delta^{13}C$ values from the Bothnian Bay fish samples (Table 2) used for independent validation of the models. The solid line shows the 1:1 ratio for observed and predicted values.

$\delta^{13}C_{treated}$–$\delta^{13}C$ and C:N ratio was found, with different invertebrate groups randomly distributed (Fig. 4).

MODEL VALIDATION FOR FISH TISSUE

All three models were validated for fish tissue using an entirely independent data set from the northern Baltic Sea (Table 2). Within this data set, untreated salmon had an average C:N ratio > 5, while the rest of the species had an average untreated C:N ratio < 3·5. These C:N ratios are representative of many fish; only extremely oily fish species such as eel exhibit higher values.

In the validation, the modified lipid-normalization model developed in the current study provided the best prediction for the observed lipid-extracted data. When observed (lipid-extracted) vs. predicted (lipid-normalized) data were plotted (Fig. 5), the data points derived from our modified model followed the line of equality closer than those derived from either of the other two models. Our modified model also had the best modelling efficiency (0·913). The modelling efficiency for the Alexander *et al*. (1996) model was 0·895 but the original McConnaughey & McRoy (1979) model resulted in 2‰ underestimates and therefore modelling efficiency was very low (–0·288).

EFFECTS OF LIPID-NORMALIZATION ON MIXING MODEL OUTPUTS

The proportion of each prey species in the Baltic ringed seal diet given by the IsoSource program was heavily dependent on whether prey and/or predator $\delta^{13}C$ values were lipid-normalized (Table 3). In the first scenario, in which both predator and prey species tissues remained un-normalized, the maximum projected contribution of herring to the seal diet was least (22%). The maximum contribution of other prey species ranged from 43% to 81%. In the second scenario, in which both ringed seal and its prey were lipid-corrected, all prey species had similar projected contributions to ringed seal diet. The third scenario, in which only prey was lipid-corrected, gave the most distinctive proportions of prey species in the seal diet. Salmon and *S. entomon* were totally absent from the diet and eelpout contributed around half of the total diet. The minimum projected proportion of herring (33%) was also much higher than in the other scenarios. In the fourth scenario, in which only predator was lipid-corrected, herring again had only minor importance in the seal diet.

Discussion

Natural abundance stable isotope tracers are often used to help elucidate food web structure. Such information has practical applications in evaluating resource management issues, assessing the impacts of introduced species and determining the effects of anthropogenic inputs of nutrients and pollutants. As these applications become more widespread and increasingly sophisticated, more care needs to be paid to procedures such as how and when to compensate results for variations as a result of the lipid content of tissues, especially when such compensations are not made empirically. Thus there is a need for further evaluation of the precision and applicability of indirect lipid-normalization approaches.

The McConnaughey & McRoy (1979) lipid-normalization model (equation 1) overestimated lipid content potentially attributable to differences in lipid-extraction procedures or to differences in the chemical composition of organisms analysed. Schlechtriem,

Table 3. Mean proportions and 1–99th percentile ranges of the percentage frequency distributions of five food sources in the Baltic ringed seal diet calculated using IsoSource program (Phillips & Gregg 2003) for four different scenarios: (i) neither prey nor ringed seal $\delta^{13}C$ values lipid-corrected (No treatment); (ii) both prey and ringed seal $\delta^{13}C$ values lipid-corrected (All treated); (iii) only prey $\delta^{13}C$ values lipid-corrected (Prey treated); (iv) only ringed seal $\delta^{13}C$ values lipid-corrected (Predator treated). Prey data were corrected for trophic fractionation ($\delta^{13}C = 1{\cdot}3$, $\delta^{15}N = 2{\cdot}4$) prior to input to the IsoSource program

	No treatment		All treated		Prey treated		Predator treated	
Prey species	Mean	1–99 percentile	Mean	1–99 percentile	Mean	1–99 percentile	Mean	1–99 percentile
Herring *Clupea harengus membras* L.	9·2%	0–22	17·0%	0–48	35·9%	33–38	3·8%	0–11
Salmon *Salmo salar* L.	18·2%	0–46	22·7%	6–41	0·0%	0–0	19·9%	0–47
Eelpout *Zoarces viviparous* (L.)	26·3%	0–72	22·7%	0–65	54·2%	46–62	26·1%	0–73
Smelt *Osmerus eprlanus* (L.)	28·0%	0–81	21·5%	0–62	9·9%	1–21	26·1%	0–81
Isopod *Saduria entomon* (L.)	18·4%	0–43	16·1%	1–32	0·0%	0–0	24·1%	3–46

Focken & Becker (2003) tested the effect of extraction methods on total lipid content and $\delta^{13}C$ values of different organisms and found only small differences. They concluded that such differences had no biological relevance, except in the case of diatoms. For our test samples, equation 1 overestimated lipid content by an average of only 3·47%, which would lead to ecologically irrelevant (about 0·2‰) overestimation of final $\delta^{13}C$ values. However, this has no impact on our model, which is derived empirically from a direct fit to C:N ratios.

In the original McConnaughey & McRoy (1979) model, all animals are normalized to a C:N ratio of 4 even if the tissue C:N ratio is < 4, as is very often the case. The $\delta^{13}C$ values of such animals are then more ^{13}C depleted after normalization, even though normalization should increase $\delta^{13}C$ values, as does chemical lipid removal. Our modified model normalizes all the values to a C:N ratio of 3·003. This is very close to the average lipid-extracted C:N values of all our fish species (3·083), suggesting that the model does indeed normalize $\delta^{13}C$ values of tissue to zero lipid content, allowing comparison with chemically lipid-extracted values. This is particularly important in studies that depend on data from samples that have been chemically lipid-extracted and data are normalized for lipids using a modelling procedure.

Our modified model provided a better fit with the chemically lipid-extracted fish data than either the original model of McConnaughey & McRoy (1979) or that of Alexander *et al*. (1996), in which the curvatures were clearly too gentle. The curvature is defined by the isotopic difference between protein and lipid in the tissue (parameter *D*), which was 6‰ in both models. McConnaughey (1978) showed how different *D*-values affect model curvature, but chose to use a value of 6‰ because this was representative of literature values and because more direct estimates were unavailable at that time. Our modified model suggests a better value for *D* to be about 7‰. Although there is still a lack of published information on isotopic differences between protein and lipid fractions in fish muscle, measured differences between lipid-free matter (mostly protein) and the lipid fraction in tilapia and carp carcasses are between 5·5% and 7·3‰ (Focken & Becker 1998; Schlechtriem, Focken & Becker 2003; Gaye-Siessegger *et al*. 2004a,b), showing that the iteratively re-estimated *D*-value in our modified model is realistic.

Our modified model provides a good fit to data from a wide variety of fish species from three different habitats and includes both lipid-rich and lipid-poor species. In the model development we used some samples from extremely oily fish, such as salmon and particularly freshwater eels, which had remarkably high C:N ratios. This makes the new model potentially applicable to a wider variety of fish, giving very good estimates of lipid-normalized $\delta^{13}C$ values. However, as the data used for independent validation of the model encompassed a more restricted range of C:N ratios, some caution should still be exercised if applying the model to samples with very high C:N ratios. In fact, the model could be easily 'fine-tuned' for any individual fish species by adjusting parameter *D*, if the isotopic difference between protein and lipid fractions in the muscle of that particular species were known.

Several authors have shown a negative relationship between $\delta^{13}C$ of untreated invertebrates and their C:N ratio (Rau, Hopkins & Torres 1991; Kline 1997; Rolff & Elmgren 2000), which has been assumed to be a consequence of increasing lipid content in the tissue. However, this assumption has never been properly verified and is not supported by our results. Even though for fish there was a very clear dependence of the $\delta^{13}C$ difference between lipid-extracted and untreated samples on the C:N ratio, no such relationship existed for our invertebrate data. There are several potential reasons for this. Stable isotope analysis of small invertebrates commonly uses whole organisms, which in crustaceans include significant amounts of chitin from the exoskeleton. Pure chitin has a C:N ratio of 6·86 (Schimmelmann & DeNiro 1986) compared with a protein value of close to 3. The average C:N ratio of invertebrates after lipid-extraction was *c.* 4, so it seems probable that non-lipid body constituents like chitin increase the C:N

ratios of the organisms. In addition, many invertebrates, including the chironomid larvae in our study, store energy as glycogen. Variations in the glycogen content can lead to large differences in C:N ratios but little change in $\delta^{13}C$ after extraction of the sample with organic solvents (DeNiro & Epstein 1978; McConnaughey 1978). Hence whole invertebrates containing appreciable quantities of chitin or non-lipid energy stores such as glycogen can have C:N ratios equivalent to those of lipid-rich fish muscle samples but would not produce an equivalent change in $\delta^{13}C$ after chemical lipid-extraction or after lipid-normalization by the modelling procedure.

However, the presence of chitin or glycogen cannot explain the large differences between $\delta^{13}C_{treated}$–$\delta^{13}C$ values of invertebrates, even when the C:N ratios of untreated samples remained rather constant (e.g. zooplankton in Fig. 4). Sato, Sasaki & Fukuchi (2002) demonstrated how mobilization of some lipid components from over-wintering copepods altered total lipid $\delta^{13}C$ values, while $\delta^{13}C$ values of other body constituents remained constant. This could account for the present results, as the zooplankton samples used in this study were collected at different times of the year. The high potential temporal (and spatial) variation in lipid $\delta^{13}C$ values makes lipid-normalization procedures based on C:N ratios impracticable for such invertebrates, because the difference between the isotopic values of lipid and protein fractions (parameter D) should be measured each time the model is applied. In fact, for zooplankton, Matthews & Mazumder (2005) found lipid-normalized $\delta^{13}C$ values derived from the original McConnaughey & McRoy (1979) model to be rather sensitive to different D-values. Hence we strongly advise that lipid-normalization procedures based on C:N ratios should not be used for invertebrates before further investigations have been carried out. If lipid correction is necessary, a chemical lipid-extraction procedure should be used.

It is clear that at present different practices are being followed with regard to correcting $\delta^{13}C$ values of animal tissues for lipid content. The IsoSource mixing model outputs for our different scenarios clearly demonstrated that lipid-normalization can have a considerable effect on estimates of dietary source proportions. This is partly because of differences in C:N ratios of prey species. The effect of normalization is largest in the case of oily fish prey species, such as salmon, with particularly high C:N ratios. Phillips & Koch (2002) emphasized the problem of unbalanced concentrations of carbon and nitrogen between dietary sources in the use of mixing models and recommended using concentration-weighted models to overcome it. To find a unique solution such models require n isotopes and $n + 1$ sources (Phillips & Koch 2002), which happens rarely. Indeed, our data had $n + 3$ sources and therefore was not applicable for concentration-weighted modelling. In such situations it would appear prudent to remove components such as lipids with skewed elemental composition.

Stable isotope analyses coupled with mixing models are being used increasingly to evaluate ecological management issues and questions. For example, the approach can help quantify the impact of predators on endangered prey populations and contribute to debate on the need to cull the predators (Bearhop *et al*. 1999), or can be used to assess the impact of different diet sources on contaminant accumulations (Power *et al*. 2002). In such studies it is essential to be aware that the output from mixing models (Phillips & Gregg 2003; Lubetkin & Simenstad 2004) is highly sensitive to whether or not carbon isotope ratios of predators and/or prey have been corrected for lipid content. We strongly recommend using concentration-weighted models if C:N ratios vary substantially between modelled species, and mixing models that do not allow for concentration dependency should be interpreted with caution. However, as differential digestibility of carbon and nitrogen among species may alter these ratios after ingestion, this should also be taken into account (Koch & Phillips 2002).

In conclusion, the widely used lipid-normalizing methods based on C:N ratios appear to be conceptually sound for vertebrate muscle tissue but we have demonstrated that lipid-normalization does not work for all species or tissue types. Therefore, we recommend that any lipid-normalization method should be tested before applying it to a particular tissue. Our modified lipid-normalization model generated estimates very close to values from chemical lipid-extraction for various lipid-poor and lipid-rich fish species from different habitats, so the model should be generally applicable to fish muscle samples. When proven applicable, our lipid-normalization model is a cost-effective way to remove the effects of lipid from large numbers of samples. It also makes it possible to correct existing $\delta^{13}C$ data from samples that have not been lipid-extracted, provided C:N ratios are available.

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