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Lipid extraction has little effect on the $\delta^{15}N$ of aquatic consumers

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Abstract

Proper application of stable isotopes (e.g., $\delta^{15}N$ and $\delta^{13}C$) to food web analysis requires an understanding of all nondietary factors that contribute to isotopic variability. Lipid extraction is often used during stable isotope analysis (SIA), because synthesized lipids have a low $\delta^{13}C$ and can mask the $\delta^{13}C$ of a consumer's diet. Recent studies indicate that lipid extraction intended to adjust $\delta^{13}C$ may also cause shifts in $\delta^{15}N$, but the magnitude of and reasons for the shift are highly uncertain. We examined a large data set (n = 854) for effects of lipid extraction (using Bligh and Dyer's [1959] chloroform-methanol solvent mixtures) on the $\delta^{15}N$ of aquatic consumers. We found no effect of chemically extracting lipids on the $\delta^{15}N$ of whole zooplankton, unionid mussels, and fish liver samples, and found a small increase in fish muscle $\delta^{15}N$ of ~0.4‰. We also detected a negative relationship between the shift in $\delta^{15}N$ following extraction and the C:N ratio in muscle tissue, suggesting that effects of extraction were greater for tissue with lower lipid content. As long as appropriate techniques such as those from Bligh and Dyer (1959) are used, effects of lipid extraction on $\delta^{15}N$ of aquatic consumers need not be a major consideration in the SIA of food webs.

Stable isotope analysis (SIA) using $\delta^{15}N$ and $\delta^{13}C$ is increasingly being used to infer trophic structure and energy flow in food webs (Peterson and Fry 1987; Thompson et al. 2005). $\delta^{15}N$ is commonly used to estimate food chain length and trophic position due to its predictable trophic enrichment, which is the difference in $\delta^{15}N$ between a consumer and its diet (Minagawa and Wada 1984). Meta-analyses show that trophic enrichment of $\delta^{15}N$ (Δ_N) is variable, with estimates of mean cross-taxa enrichment between 2.0‰ and 3.4‰ (SD = 1.0; Post 2002; McCutchan et al. 2003; Vanderklift and Ponsard 2003). Several factors lead to variation in Δ_N, including nitrogen metabolism, food quality, and temperature (McCutchan et al. 2003; Vanderklift and Ponsard 2003; Sweeting et al. 2007). In addition, certain methodological treatments can affect the $\delta^{15}N$ of biological tissues, including preservation, acidification, and lipid-extraction (Gannes 1997; Kaehler and Pakhomov 2001; Arrington and Winemiller 2002; Feuchtmayr and Grey 2003; Ng et al. 2007). For $\delta^{15}N$ to be a useful metric of trophic position and variation, it is critical to understand factors aside from trophic enrichment that can influence isotopic values.

In this paper, we focus on the effects of lipid extraction on the $\delta^{15}N$ of aquatic organisms. Lipid extraction is commonly used in stable isotope analysis to "correct" the $\delta^{13}C$ of consumers to better reflect the $\delta^{13}C$ of their diets (Matthews and Mazumder 2005; Post et al. 2007). There is preferential incorporation of ^{12}C into lipids during synthesis, resulting in the carbon in those lipids being isotopically lighter (~6‰) than dietary carbon (DeNiro and Epstein 1977). Thus, if a tissue is lipid-rich, many researchers will choose to extract lipids under the rationale that the $\delta^{13}C$ of the lipid-free fraction should better match the $\delta^{13}C$ of the organism's diet (e.g., Beaudoin et al. 1999; Post 2002). Alternatively, others use a numerical correction (McConnaughey and McRoy 1979; Kiljunen et al. 2006) to normalize $\delta^{13}C$ values to a constant lipid content that is inferred by C:N ratios (e.g., Kline 1999; Power et al. 2002). Though lipid extraction is a common procedure in SIA, some studies suggest it could cause shifts in $\delta^{15}N$ and significantly

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Table 1. Samples used to analyze the effect of lipid extraction on $\delta^{15}N$ in aquatic consumer tissues, with habitat (F = freshwater, B = brackish water, M = marine) and location of collection

Tissue	Species	Common	Habitat	Collection location
Fish liver	*Abramis brama*	Carp bream	F	Plußsee, Germany*
	Anguilla anguilla	European eel	F	Plußsee, Germany*
	Perca fluviatilis	European perch	F	Plußsee, Germany*
	Rutilus rutilus	Roach	F	Plußsee, Germany*
	Gymnocephalus cernuus	Ruffe	F	Plußsee, Germany*
	Coregonus lavaretus	Common whitefish	F,B	Finland*
Fish muscle	*Anguilla anguilla*	European eel	F,B	Screebe, Ireland*
	Perca fluviatilis	European perch	F	Lough Neagh, Northern Ireland*
	Coregonus autumnalis	Arctic cisco	F	Lough Neagh, Northern Ireland*
	Rutilus rutilus	Roach	F	Plußsee, Germany*
	Gasterosteus aculeatus	Threespine stickleback	F	Kennedy Lake, Canada†
	Coregonus lavaretus	Common whitefish	F,B	Finland*
	Sebastes caurinus	Copper rockfish	M	Kyuquot Sound, Canada‡
	Sebastes melanops	Black rockfish	M	Barkley Sound, Canada‡
body	Unionidae	Mussel	F	Kennedy Lake, Canada†
foot	Unionidae	Mussel	F	Kennedy Lake, Canada†
whole	*Leptodiaptomus tyrelli*	Calanoid Copepod	F	Sooke reservoir, Canada†
whole	N/A	Bulk Zooplankton	F	Plußsee, Germany*

Symbols indicate which authors contributed each sample:
*CH, JG, and TS
†BM and AM
‡RM

affect the interpretation of food web structure (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Murry et al. 2006).

Tests for changes in $\delta^{15}N$ of tissues as a result of lipid extraction have yielded mixed results. Pinnegar and Polunin (1999) detected an average increase of $\delta^{15}N$ of 0.78‰ for muscle and 0.61‰ for liver of juvenile Rainbow Trout following lipid extraction. Sotiropoulos et al. (2004) found small positive shifts in $\delta^{15}N$ of lipid-extracted muscle tissues of three freshwater fish species (0.3-0.6‰, $n = 36$) and much larger positive shifts for whole juvenile fathead minnow (2.8‰, $n = 19$). Sweeting et al. (2006) found increases in $\delta^{15}N$ in lipid-extracted liver and muscle tissue of European sea bass (0.79‰, $n = 93$). Murry et al. (2006) extracted lipids from muscle tissue of 9 freshwater fish species and found much larger increases in $\delta^{15}N$ than previous studies (1.59‰, $n = 67$). Such large shifts in $\delta^{15}N$ would have considerable impacts on estimates of trophic position, and could pose significant problems for comparing trophic structure across studies and ecosystems. Concern over effects of lipid extraction has led to suggestions that separate, unextracted sample aliquots should be analyzed for $\delta^{15}N$, though this would substantially increase (i.e., double) analysis costs (e.g., Sotiropoulos et al. 2004). Due to the implications of the recent findings of Murry et al. (2006) and the current state of uncertainty in the literature, we addressed the effect of lipid extraction on $\delta^{15}N$ of aquatic consumers using a large data set ($n = 854$). We extracted lipids from tissues of multiple fish and invertebrate species and found little to no effect on $\delta^{15}N$, depending on tissue type.

Materials and procedures

We tested for effects of lipid extraction on $\delta^{15}N$ values using stable isotope signatures of freshwater, brackish and marine fishes, and freshwater invertebrates collected at multiple locations in Europe and North America (Table 1). Lipids were extracted from 854 samples, including skinless dorsal muscle tissue from 8 fish species ($n = 325$), liver tissue from 6 fish species ($n = 466$), unionid mussels (foot muscle tissue [$n = 7$] and whole bodies [$n = 8$]), whole bodies of calanoid copepods ($n = 19$), and bulk zooplankton samples ($n = 49$). In many cases, sampling protocols are described elsewhere (Matthews and Mazumder 2003; Harrod et al. 2005; Kiljunen et al. 2006; Harrod and Grey 2006; Harrod et al. unpubl. data). *Perca fluviatilis* and *Coregonus autumnalis* were collected from Lough Neagh, Northern Ireland (54°35′N, 6°25′W) using bottom and surface set multipanel gillnets (Harrod and Griffiths unpubl. data). *Gasterosteus aculeatus* and unionid mussels were collected from Kennedy Lake, British Columbia, Canada (49°05′58.41_N,125°35′32.04_W). *G. aculeatus* were caught overnight in minnow traps and mussels were collected by dipnet, then samples were frozen on dry ice. Adult *Sebastes caurinus* (19-45 cm total length) were collected from Kyuquot Sound (50°00′N, 127°27′W) and juvenile *Sebastes melanops* (40-60 mm total length) were collected from Barkley Sound (48°53′N, 125°27′W), both on the west coast of Vancouver Island, British Columbia, Canada.

Tissues were freeze-dried or oven-dried, then ground to powder using mortar and pestle, a freezer mill (6750 SPEX;

Table 2. Effects of lipid extraction on tissue $\delta^{15}N$ of aquatic organisms

Tissue	Species	*n*	C:N Mean	$\delta^{15}N_R$ Mean	St. Dev.	$\delta^{15}N_{LE-R}$ Mean	St. Dev.	Means *P*	Variances *F*	Variances *P*
Fish liver	*A. brama*	18	4.23	9.61	0.44	0.19	0.56	0.17	1.47	0.43
	A. anguilla	17	4.05	10.11	0.70	–0.02	0.61	0.88	1.37	0.53
	P. fluviatilis	99	4.07	7.90	0.58	–0.14	0.49	0.007*	1.06	0.77
	R. rutilus	59	5.01	8.29	0.57	0.06	0.37	0.25	0.53	0.02*
	G.cernuus	28	3.80	7.33	0.83	0.18	0.39	0.021*	1.07	0.86
	C. lavaretus	242	3.89	7.50	1.46	0.19	0.28	<0.01*	1.06	0.65
Fish muscle	*A. anguilla*	107	3.14	12.06	1.08	0.38	0.26	< 0.01*	0.92	0.69
	P. fluviatilis	15	2.81	18.61	0.57	0.54	0.77	0.016*	0.92	0.87
	C. autumnalis	16	2.96	19.94	0.33	0.36	0.14	<0.01*	1.51	0.44
	R. rutilus	32	2.87	18.43	0.62	0.47	0.30	<0.01*	0.7	0.33
	G.aculeatus	16	4.14	8.99	0.52	0.06	0.12	0.07	0.9	0.84
	C. lavaretus	121	2.95	8.09	1.09	0.38	0.34	<0.01*	1.12	0.53
	S. caurinus	10	3.22	16.03	0.52	1.00	0.16	<0.01*	1.04	0.95
	S. melanops	10	3.37	11.29	0.30	0.90	0.12	<0.01*	1.47	0.57
body	Mussel	8	5.22	2.34	0.31	–0.07	0.18	0.28	0.86	0.85
foot	Mussel	7	4.69	2.30	0.25	0.03	0.12	0.49	0.42	0.33
whole	Copepod	19	12.03	6.27	0.73	0.05	0.22	0.3	0.92	0.86
whole	Bulk Zooplankton	49	N/A	5.43	3.08	0.04	0.78	0.75	0.87	0.87

Mean C:N ratio and means and standard deviations are shown for raw nitrogen isotope values ($\delta^{15}N_R$) and for the difference between lipid-extracted and raw $\delta^{15}N$ ($\delta^{15}N_{LE-R}$) are shown. Mean $\delta^{15}N_{LE}$ and $\delta^{15}N_R$ were compared using paired *t* tests, and variances were compared using *F*-tests (F = Variance of $\delta^{15}N_{LE}$ / Variance of $\delta^{15}N_R$).
*Significant *P* values.

CertiPrep Inc.), or a digital Touchpad Amalgamator (Henry Schien Inc.). Samples were divided into two aliquots: one was untreated (hereafter $\delta^{15}N_R$) and lipids were extracted from the other ($\delta^{15}N_{LE}$). Extractions followed the Bligh and Dyer (1959) protocol, with chloroform, methanol, and water in a 4:2:1 ratio. Samples analyzed by B.M., A.M. and R.M. (see Table 1) were extracted over <15 min following Bligh and Dyer (1959), while samples analyzed by C.H., T.S., and J.G. were subjected to an additional extraction period of 24 h. $\delta^{15}N$ and C:N were determined for each aliquot using a Eurovector elemental analyser coupled to a Micromass Isoprime continuous flow mass spectrometer (Micromass) for samples contributed by C.H., T.S., and J.G.; a Finnigan Delta Plus Advantage isotope ratio mass spectrometer (Finnigan MAT) for samples contributed by B.M. and A.M.; and a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer (PDZ Europa) for samples contributed by R.M. In most cases, replicated samples of fish tissue were analyzed as laboratory standards to assess measurement error, with standard deviations of replicated samples of 0.14‰ (data contributed by B.M. and A.M.; see Table 1) and 0.3‰ (data contributed by C.H., T.S., and J.G.). Where replicated measurements were unavailable, the long-term precision of the instruments (< 0.3‰) were used as estimates of the measurement precision.

We grouped our data based on species and tissue type, and analyzed each of the 18 groups separately. For each group, we used a paired *t* test to test whether the average difference between the raw ($\delta^{15}N_R$) and lipid-extracted ($\delta^{15}N_{LE}$) nitrogen isotope ratios of consumers significantly differed from zero. Following previous investigators (e.g., Sotiropoulos et al. 2004; Murry et al. 2006; Sweeting et al. 2006), we also used *F*-tests to assess whether lipid extraction affects the variance of $\delta^{15}N$. For fish muscle tissue, we used a linear regression to test whether the magnitude of the lipid extraction effect was consistent across the range of $\delta^{15}N$ of the tissues used (~5‰-20‰). We also evaluated the effect of differences in lipid content of fish muscle tissue with a linear regression of the effect of extraction ($\delta^{15}N_{LE}$ - $\delta^{15}N_R$) against the pre-extraction C:N ratio. All statistical analyses were carried out in the R environment (R Development Core Team 2006).

Assessment

Generally, shifts in mean $\delta^{15}N$ following lipid extraction were small in magnitude (range –0.14‰ to 1.00‰), and sample variance was unaffected (Table 2). Fish muscle tissue showed a small but consistent enrichment, with 7 of 8 species showing a mean increase in $\delta^{15}N$ between 0.36‰ and 1.00‰ ($P < 0.02$). Fish liver tissue showed no consistent effect ($-0.14 < \delta^{15}N_{LE-R} < 0.19$), while mussels, copepods, and bulk zooplankton showed no significant change in $\delta^{15}N$ with lipid extraction ($P > 0.25$). Lipid extraction did not affect variance in $\delta^{15}N$ in 17 of 18 comparisons; liver tissue of *Rutilus rutilus* showed a significant decrease in variance following extraction ($F_{58,58} = 0.53$, $P = 0.016$).

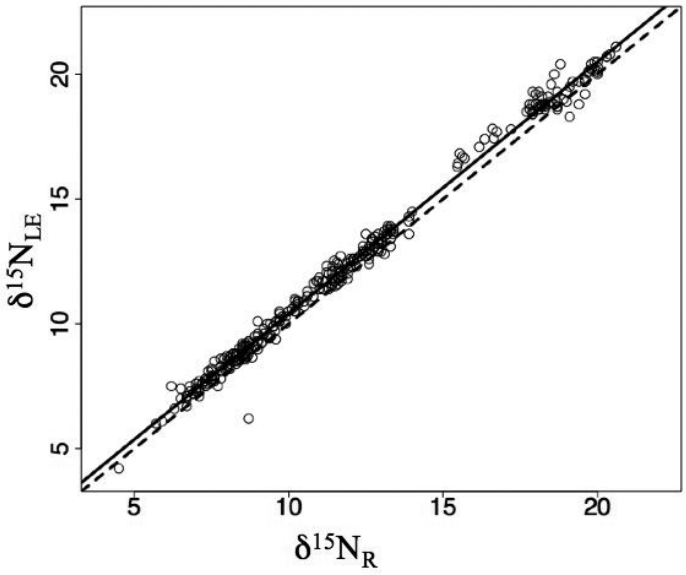

Fig. 1. Relationship between lipid-extracted ($\delta^{15}N_{LE}$) and raw ($\delta^{15}N_R$) nitrogen isotope values in fish muscle tissue. Dashed line shows 1:1 relationship (no effect of extraction), whereas solid line shows linear regression fit line with equation $\delta^{15}N_{LE} = (0.29 \pm 0.06\ SE) + \delta^{15}N_R \times (1.01 \pm 0.005\ SE)$.

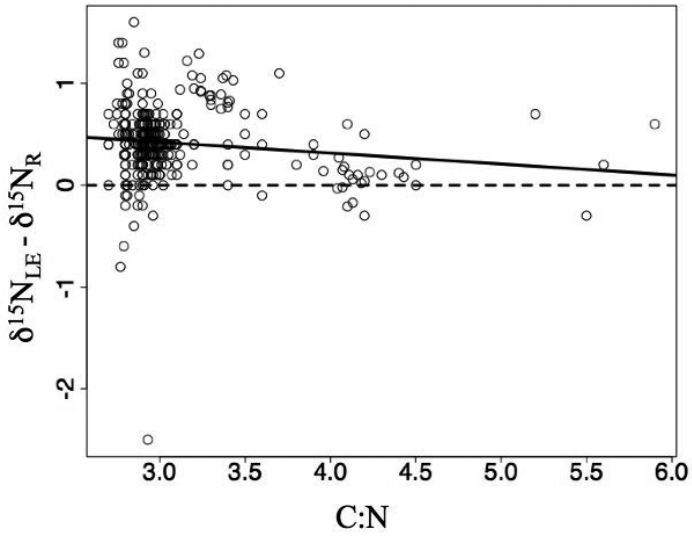

Fig. 2. Relationship between effect of lipid extraction on $\delta^{15}N$ in fish muscle tissue ($\delta^{15}N_{LE} - \delta^{15}N_R$) and tissue C:N ratio. Dashed line indicates $\delta^{15}N_{LE} - \delta^{15}N_R = 0$ (no effect of extraction) whereas solid line shows linear regression fit line with equation $\delta^{15}N_{LE} - \delta^{15}N_R = (0.75 \pm 0.13\ SE) - C{:}N \times (0.108 \pm 0.04\ SE)$; $F_{1,325} = 6.22$, $P = 0.01$.

The slope of the regression equation describing the relationship between $\delta^{15}N_{LE}$ and $\delta^{15}N_R$ in fish muscle tissue is very close to one (1.01 ± 0.05 SE), indicating that the effect of extraction is consistent across the large range of $\delta^{15}N$ values in the analysis (4.5‰-20.6‰; Fig. 1). The estimated intercept in the equation is 0.29 ($P < 0.001$), slightly below the typical effects observed using paired *t* tests (Table 2). There is also a weak but significant negative relationship between $\delta^{15}N_{LE\text{-}R}$ and C:N ratio (Fig. 2; $F_{1,305} = 6.22$, $P = 0.01$), suggesting that extraction was more likely to affect $\delta^{15}N$ of muscle tissue with low lipid content (i.e., low C:N).

Discussion

We examined a large dataset ($n = 854$) of $\delta^{15}N$ values for a range of aquatic consumers and found small and generally negligible effects of lipid extraction on $\delta^{15}N$. The only consistent effect that we identified was an increase of 0.36‰-1.00‰ in the mean $\delta^{15}N$ of fish muscle tissue following extraction. The magnitude of these increases is comparable with those observed by Sotiropoulos et al. (2004) and Pinnegar and Polunin (1999), and substantially lower than the increases in $\delta^{15}N$ reported by Murry et al. (2006). Unlike Sweeting et al. (2006), we found that the magnitude of the shift in $\delta^{15}N$ for muscle tissue was consistent across a large range of $\delta^{15}N_R$ values. Our results are consistent with previous data showing that lipid extraction does not change the variance in $\delta^{15}N$ (Sotiropoulos et al. 2004; Murry et al. 2006; Sweeting et al. 2006). On the basis of these results, the use of lipid-extracted samples for $\delta^{15}N$ determination should not significantly affect interpretation of food web structure.

Discrepancies between our results and recent reports of large increases in $\delta^{15}N$ following lipid extraction (Murry et al. 2006) may result from differences between extraction protocols. Studies that have applied chloroform-methanol solvent mixtures following Bligh and Dyer (1959), including the present study, have generally found relatively small effects of lipid extraction on $\delta^{15}N$ (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Sweeting et al. 2006), whereas Murry et al. (2006) used a dichloromethane-methanol solvent mixture. To our knowledge, these methods have not been compared directly to assess potential differences in efficacy or specificity. Solvents that more efficiently extract lipid also extract more nonlipid polar molecules such as structural proteins, which could lead to different shifts if proteins differ in $\delta^{15}N$ (Sweeting et al. 2006). Individual amino acids can differ substantially in their $\delta^{15}N$ (McClelland and Montoya 2002; Schmidt et al. 2004), so differences in amino acid composition could cause proteins in different tissues to be extracted with different efficiencies, potentially leading to variation in the effect of extraction on $\delta^{15}N$.

Whereas the mechanisms that increase $\delta^{13}C$ following lipid extraction are well understood (DeNiro and Epstein 1977), little is known about the processes that sometimes lead to positive shifts in $\delta^{15}N$ with lipid extraction. Two proposed mechanisms for this shift are discussed by Murry et al. (2006). As described above, an extraction of polar proteins in association with membrane lipids could increase $\delta^{15}N$, but only if lipid-associated proteins have a substantially lower $\delta^{15}N$ than other nitrogenous compounds. In this case, we would expect $\delta^{15}N_{LE\text{-}R}$ and lipid content (or C:N) to be positively correlated, because

lipid extraction would remove proportionally more nitrogen with low $\delta^{15}N$. However, we found a significant, albeit weak negative relationship between $\delta^{15}N_{LE\text{-}R}$ and C:N of fish muscle (Fig. 2), a pattern previously reported by Sweeting et al. (2006). In a similar vein, whole organisms and fish liver tissue tend to have higher C:N and lower $\delta^{15}N_{LE\text{-}R}$ (Table 2). As a result, we do not believe removal of lipid-bound proteins is a likely mechanism associated with positive shifts in $\delta^{15}N$ following lipid extraction. The second proposed mechanism for $\delta^{15}N$ shifts is the leaching of nitrogenous metabolites (principally ammonia and ammonium) remaining in the tissue (Murry et al. 2006). As consumers preferentially retain ^{15}N during transamination and deamination, the waste products from protein metabolism have lower $\delta^{15}N$ and their removal during lipid extraction should increase the $\delta^{15}N$ of the remaining tissue (Bearhop et al. 2000). Linking variation in tissue composition (Persson et al. 2007) with compound-specific $\delta^{15}N$ measurements (McClelland and Montoya 2002) would be useful for determining the plausibility of these mechanisms.

Comments and recommendations

Our results show that chloroform-methanol lipid extraction has little overall effect on the $\delta^{15}N$ of aquatic consumers. The small effects we did observe differ depending on the type of tissue analyzed and its C:N content. Because shifts in $\delta^{15}N$ were typically < 0.5‰ and similar in magnitude to the analytical precision, lipid extraction is unlikely to affect the inference of food web structure using SIA. If lipid extraction is desirable for $\delta^{13}C$ analysis, it should not be necessary to double analysis costs by using separate sample aliquots to measure $\delta^{15}N$ and $\delta^{13}C$ (Sotiropoulos et al. 2004). In fact, the weak negative relationship between $\delta^{15}N_{LE\text{-}R}$ and C:N suggests that the effects of lipid extraction may be less when extraction is most likely to be necessary (i.e., in lipid-rich tissues). Of greater concern for comparisons across studies is the possible difference in solvent effects during lipid extraction. Until further comparisons of solvents have been carried out, we recommend that chloroform-methanol extractions based on the original protocols of Bligh and Dyer (1959) be employed to minimize shifts in $\delta^{15}N$. An alternative approach for the study of fish muscle tissues that ensures that $\delta^{15}N$ values remain unchanged is to use a mathematically derived correction factor to remove any effect of lipids on $\delta^{13}C$ (Kiljunen et al. 2006; Post et al. 2007).

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