

# Applications, Considerations, and Sources of Uncertainty When Using Stable Isotope Analysis in Ecotoxicology

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Stable isotope analysis (SIA) has become a powerful tool for ecotoxicologists to study dietary exposure and biomagnification of contaminants in wild animal populations. The use of SIA in ecotoxicology continues to expand and, while much more is known about the mechanisms driving patterns of isotopic ratios in consumers, there remain several considerations or sources of uncertainty that can influence interpretation of data from field studies. We outline current uses of SIA in ecotoxicology, including estimating the importance of dietary sources of carbon and their application in biomagnification studies, and we present six main considerations or sources of uncertainty associated with the approach: (1) unequal diet-tissue stable isotope fractionation among species, (2) variable diet-tissue stable isotope fractionation within a given species, (3) different stable isotope ratios in different tissues of the animal, (4) fluctuating baseline stable isotope ratios across systems, (5) the presence of true omnivores, and (6) movement of animals and nutrients between food webs. Since these considerations or sources of uncertainty are difficult to assess in field studies, we advocate that researchers consider the following in designing ecotoxicological research and interpreting results: assess and utilize variation in stable isotope diet-tissue fractionation among animal groups available in the literature; determine stable isotope ratios in multiple tissues to provide a temporal assessment of feeding; adequately characterize baseline isotope ratios; utilize stomach contents when possible; and assess and integrate life history of study animals in a system.

## Introduction

Naturally occurring stable isotope ratios of carbon ( $^{13}\text{C}/^{12}\text{C}$  or  $\delta^{13}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$  or  $\delta^{15}\text{N}$ ), sulfur ( $^{34}\text{S}/^{32}\text{S}$  or  $\delta^{34}\text{S}$ ), hydrogen ( $^2\text{H}/^1\text{H}$  or  $\text{D}/\text{H}$  or  $\delta\text{D}$ ), and oxygen ( $^{18}\text{O}/^{16}\text{O}$  or  $\delta^{18}\text{O}$ ) have become powerful and popular tools to trace organic matter sources in ecosystems. Isotopic ratios, expressed in delta notation, are calculated following the formula:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad (1)$$

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where X is the heavier isotope (e.g.,  $^{13}\text{C}$ ),  $R_{\text{sample}}$  is the raw ratio of the heavy to light isotope in the sample, and  $R_{\text{standard}}$  is the raw ratio of the heavy to light isotope in an internationally accepted standard. These standards include variations of Peedee belemnite (PDB) carbonate for  $\delta^{13}\text{C}$ , atmospheric nitrogen (AIR) for  $\delta^{15}\text{N}$ , Canyon Diablo troilite (CDT) for  $\delta^{34}\text{S}$ , and standard mean ocean water (SMOW) for  $\delta\text{D}$  and  $\delta^{18}\text{O}$  (1).

The use of stable isotope analysis (SIA) in studies of animal ecology rests on two central tenets: (1) stable isotope ratios in consumers are proportional to those in their putative diet, and (2) differences in isotope ratios exist among food sources available for consumers. Early laboratory studies (2, 3) established the validity of the first tenet. They reported that lab-reared consumers had stable carbon isotope ratios that were not statistically different than their food (mean difference =  $0.8 \pm 1.1\%$  S.D.), while consumers were enriched in  $^{15}\text{N}$  relative to their diet (mean difference =  $3.0 \pm 2.6\%$  S.D.). These relationships have since been corroborated in a series of literature syntheses of lab-based trials (4–6). The second tenet has been demonstrated repeatedly in a variety of field studies that showed isotopic differences among different food types, including C3 vs C4 plants (7) and organic matter originating from aquatic vs terrestrial (8), marine vs freshwater (9) and planktonic vs benthic (10–12) habitats. These two central tenets form the foundation of stable isotope ecology that is practiced today. One isotope (typically carbon) shows considerable isotopic change during its fixation by primary producers, while another isotope (typically nitrogen) shows considerable change as it is processed by consumers. Therefore, the combination of these two isotopes allows the investigation of different energy flow processes that shape the structure and function of food webs. SIA has advantages over traditional dietary analyses (e.g., gut contents) because it provides a time-integrated representation of assimilated food rather than a snapshot of recently ingested items (13).

Coincident with the increase in knowledge concerning SIA has been an expansion in its applications. One such expansion is into the field of ecotoxicology, wherein SIA is used to explain variability in contaminant concentrations of animal populations. Because of the importance of diet as a route of exposure for heavy metals, organochlorines, and other persistent contaminants (14, 15), SIA has considerably advanced the field of ecotoxicology by linking wild animal populations to their diet and ultimate contaminant source. Ecotoxicology studies that use SIA can be considered in three general categories: (1) qualitative linkages between dietary habits of animal populations and their contaminant concentrations, (2) food web biomagnification studies, and (3) quantitative assessments of habitat-specific foraging as a means of explaining biotic contaminant concentrations. The

strongest studies combine categories 2 and 3, simultaneously assessing the importance of both food chain length and underlying dietary pathway in determining contaminant concentrations in an organism. Estimating trophic position and determining underlying organic matter pathways are typically accomplished with  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , respectively, while  $\delta^{34}\text{S}$ ,  $\delta\text{D}$ , and  $\delta^{18}\text{O}$  are increasingly being used to examine larger scale animal movement patterns (16). This review will focus on how  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  have been used in the past to relate contaminant concentrations in an organism to its dietary characteristics, and will highlight six of the main considerations/sources of uncertainty when using the technique.

**Qualitative Studies.** In aquatic systems, food as opposed to water is often the major exposure route of animals to contaminants, particularly mercury (15) and hydrophobic organic contaminants such as PCBs and DDT (14). For air-breathing organisms, food is usually the only exposure route for contaminants. Thus, researchers who study contaminant fate in biota may wish to examine the diet of animal populations and may use isotope data to generate hypotheses as to why certain subpopulations or specific taxa have higher or lower contaminant concentrations than their counterparts occupying similar habitats. In these studies, contaminant concentrations are not directly and quantitatively linked to isotope ratios, rather isotope ratios are first compared statistically among taxa, followed by the analyses of inter-taxa differences in contaminant concentrations. For example, the scavenging gastropod *Cyclope neritea* had elevated  $\delta^{15}\text{N}$  when compared to other organisms in the River Po delta in Italy, and its higher trophic position was used to explain the gastropod's higher concentrations of mercury, cadmium, lead, copper, and zinc (17). Other examples of these types of statistical approaches include examinations of trace metals in marine food webs (18), organochlorines and metals in avian tissues (19–22), and mercury in riverine fishes (23). Other such studies have focused on a single species and used SIA to explain variability in contaminant concentrations within these species. They include analyses of mercury in great skua (*Catharacta skua*) chicks (24) and common terns (*Sterna hirundo*, ref 25), organochlorines in walrus (*Odobenus rosmarus*, ref 26), Atlantic salmon (*Salmo salar*, ref 27), glaucous gulls (*Larus hyperboreus*, ref 28), and Arctic fox (*Alopex lagopus*, ref 29), and metals in raccoons (*Procyon lotor*, 30).

**Biomagnification Studies.** The studies described above, while valuable in providing some explanation for the variability observed in animal contaminant concentrations, do not offer insight into ecosystem-scale patterns, and typically use SIA in a semiquantitative fashion. One of the advantages of SIA is its quantitative nature and this application has led to a movement away from categorical foraging data (31). As one such example, an initial study in eastern Canada had classified lakes based on the presence or absence of different forage species (e.g., mysids, forage fishes), assigned organisms to discrete trophic levels (TLs), and used this information to predict/describe concentrations of mercury in top predators across systems (32). Lakes with long food chains due to the presence of mysids and/or forage fishes had lake trout (*Salvelinus namaycush*) with higher mercury concentrations than lakes with short food chains lacking intermediate trophic links (33). Follow-up studies on these same systems used SIA and showed that omnivory was common in the fish species, demonstrating that  $\delta^{15}\text{N}$  is a more powerful approach than discrete TL classifications in predicting contaminant concentrations of higher order predators (34, 35).

The first study that used SIA to quantify the trophic transfer or biomagnification of contaminants through entire food webs (37) used regressions relating concentrations of poly-

chlorinated dibenzo-*p*-dioxins (PCDDs) to  $\delta^{15}\text{N}$  following

$$[\text{PCDD}] = e^{(b + m \times \delta^{15}\text{N})} \quad (2)$$

which transforms to

$$\ln [\text{PCDD}] = b + m \times \delta^{15}\text{N} \quad (3)$$

In this equation, the slope ( $m$ ) is a measure of the biomagnification of PCDD through the food web, whereas the intercept ( $b$ ) may represent the concentration of the contaminant at the base of the food web, although this requires further investigation. Recently, the  $\delta^{15}\text{N}$  values in eq 3 have been replaced with TL (see ref 38) estimates that are based on the  $\delta^{15}\text{N}$  value of an organism and are calculated using the follow equation (39):

$$\text{TL}_{\text{consumer}} = (\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{primary producer}}) / \Delta 15\text{N} + 1 \quad (4)$$

where  $\delta^{15}\text{N}_{\text{primary producer}}$  is assumed to occupy TL 1 and  $\Delta 15\text{N}$  (the "enrichment factor") represents the increase in  $\delta^{15}\text{N}$  from one TL to the next. Substituting TL into eq 3 gives the following:

$$\ln \text{ or } \log [\text{contaminant}] = b + m \times \text{TL} \quad (5)$$

and a food web magnification factor [FWMF; has also been called a trophic magnification factor (TMF)] can be calculated (40):

$$\text{FWMF} = e^m \text{ (or } 10^m) \quad (6)$$

The advantage of the FWMF over a  $\delta^{15}\text{N}$ -derived slope is that the former represents the increase in contaminant concentrations from one TL to the next averaged over the entire food web, and is analogous to a biomagnification factor for the food web (41). It is also corrected for the baseline variation in  $\delta^{15}\text{N}$  that occurs among systems (discussed in more detail below). In contrast, the slopes of the contaminant- $\delta^{15}\text{N}$  regressions quantify the increase in contaminant concentrations with each per mil  $\delta^{15}\text{N}$ , a measure that is much more abstract with respect to its application to prey-to-predator transfer of these contaminants. Furthermore, the use of TL allows unique enrichment factors for species or groups of animals to be incorporated into the FWMF estimates of trophic transfer of contaminants. More specifically, food web studies that have included birds with invertebrates, fish, and mammals can utilize different enrichment factors for these different taxa (40, 42). Both unadjusted and adjusted (for different diet-tissue  $^{15}\text{N}$  enrichment) contaminant-TL relationships can therefore be useful in assessing biomagnification within and across systems.

Over the past decade SIA has been valuable in assessing the biomagnification potential of a variety of contaminants. While certain contaminants (and congeners) increase in concentration with increasing TL (e.g., organochlorines, refs 40, 43–50; mercury, refs 43, 51–53; and rubidium and cesium, ref 54), others have shown no significant relationship or have decreased significantly when regressed against  $\delta^{15}\text{N}$  of the biota within the food web (43, 55–58). Assessing the potential for a particular contaminant to biomagnify in a lacustrine, riverine or marine food web is important because those chemicals that show limited biomagnification potential presumably pose less of a threat to the health of humans and wildlife that rely on aquatic food webs as a source of food. As a result, biomagnification potential has been advocated as a means for determining the level of regulation (i.e., limitation of releases to the environment) to assign to different industrial chemicals in bureaucratic legislation (59).

**Quantitative Assessment Of Carbon Sources.** Another factor that may affect an organism's exposure to and accumulation of contaminants is the habitat in which it forages relative to other individuals within a population. The first paper to demonstrate a link between foraging patterns and contaminants showed a relationship between a variety of contaminants in marine fishes and their tissue carbon, nitrogen, and hydrogen isotope ratios (60). Fishes with less "heavy" carbon and nitrogen isotopic ratios derived a larger proportion of their biomass from a sewage outfall; a significant negative trend was observed between organochlorines and  $\delta^{15}\text{N}$  for these species, suggesting greater exposure in fish that were connected to the sewage outfall (60).

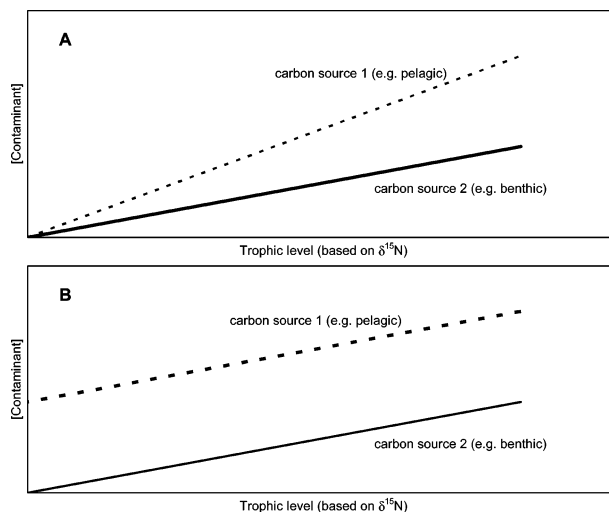
Mathematical formulas have been created to quantify source contributions to an organism using stable isotopes, assuming the dietary sources differ enough in their isotope ratios to allow separation (9). Calculation of the relative importance of two dietary sources to an animal is achieved by quantifying the isotope ratios and calculating the relative isotopic difference of the sources, and then comparing the organism's isotope ratio to one of these sources of food. In a simple two source, one isotope mixing model, dietary carbon source is calculated according to

$$P_a = (\delta^{13}\text{C}_{\text{organism}} - \delta^{13}\text{C}_b) / (\delta^{13}\text{C}_a - \delta^{13}\text{C}_b) \quad (7)$$

where  $P_a$  is the proportion of source a (e.g., pelagic carbon) in the diet, and  $\delta^{13}\text{C}_a$  and  $\delta^{13}\text{C}_b$  are the baseline carbon isotope ratios for source a and b, respectively. In this model, there is no fractionation of  $^{13}\text{C}$  through the food web (e.g., ref 61) but, if necessary, this can be accounted for by adjusting the source values by an appropriate amount (e.g., 0.8‰, ref 5).

One of the original limitations associated with these mixing equations was their inability to reflect the error around mean estimates of sources and mixtures; this problem has been overcome by the recent development of mixing model theory and software that covers a variety of scenarios for which such mixing models are appropriate. These include standard one-isotope, two-source, and two-isotope, three-source models (Isoerror, ref 62); concentration-dependent mixing models that account for the carbon and nitrogen stoichiometry of various food types (Isoconc, ref 63); and mixing models that provide probability distributions for various sources when the number of sources is much greater than the number of isotopes available for analysis (Isosource, ref 64), such as the separation of littoral, pelagic, and profundal energy sources using only  $\delta^{13}\text{C}$  in lakes. These models also conserve mass balance, a feature that was lacking in earlier models. It should also be noted that in some cases it may not be possible or necessary to assess the relative importance of different carbon sources because the food web is driven by energy from a single food source (e.g., phytoplankton, 51).

Multiple stable isotopes can be used to understand and quantify the influence of both underlying dietary pathways and trophic positioning on concentrations of contaminants in individuals and populations. For example,  $\delta^{13}\text{C}$  was used to delineate organisms connected to the benthic and pelagic food chains in Lake Malawi, Africa (48, 52). The biomagnification slope for  $\Sigma\text{DDT}$  (vs  $\delta^{15}\text{N}$ ) was higher in the pelagic than the benthic food web, resulting in higher  $\Sigma\text{DDT}$  in top predators from the former niche in the system (48). In the same lake, concentrations of mercury were also higher in the biota relying upon pelagic carbon despite similar biomagnification slopes for that contaminant between the benthic and pelagic food webs; pelagic feeders had higher Hg, most likely due to higher inputs of Hg at the base of this food web (52). Figure 1 illustrates the theoretical expectations for isotope and contaminant data under these scenarios.



**FIGURE 1. Theoretical biomagnification of contaminants in contrasting food webs with similar baselines and different slopes (A), and similar slopes and different baselines (B). Animals deriving greater than 70% of their biomass from carbon source 1 or carbon source 2 were separated using  $\delta^{13}\text{C}$ .**

Animals are assigned to one of two habitats based on  $\delta^{13}\text{C}$  (with >70% contribution as a criteria, ref 52), and contaminant concentrations plotted against TL to assess biomagnification. In Figure 1a, both habitats have similar baseline contaminant concentrations, but the slope is much steeper for biota from habitat A. In Figure 1b, the contaminant-TL slopes are the same but a higher baseline contaminant level in habitat A results in increased contaminant concentrations in all organisms from that food web. As application of this technique increases, analyses of covariance (65) can be used to assess if slopes and intercepts differ among two habitat types within one system or among systems. The importance of both TL and carbon source in determining fish Hg concentrations was also demonstrated in a sub-Arctic lake (36). Similar to the Lake Malawi food web (52), fishes that derived more energy from the pelagic zone had higher Hg than those connected to the benthic pathway (36). Stable carbon isotopes were also used to identify phytoplankton and epiphytes as two major primary food sources for consumers in the Sacramento-San Joaquin River delta (66). In that study, the authors found a significant increase in cadmium concentrations with increasing TL in the epiphyte-based food web that was not apparent when the entire food web was considered collectively (66). Separating food source pathways along with measures of TL has also been used to understand the fate of contaminants in small rivers and streams. In Swedish streams, higher PCB concentrations were found in animals associated with the detrital pathway when compared to the algal pathway (67). PCB concentrations also increased from primary producers through invertebrates to brown trout (*Salmo trutta*), suggesting that both TL and carbon sources were important in those systems (67).

Testing the influence of both factors (carbon source and TL) also allows one to make a comparison of their relative importance. For example, lipid-rich animals connected to the pelagic zone in Bow Lake, Canada had higher organochlorine concentrations than those feeding in benthic habitats, and the effect of lipid and carbon source was far more dominant than TL (estimated by  $\delta^{15}\text{N}$ ) in explaining variability in biotic concentrations (68).

### Considerations and Sources of Uncertainty

While SIA has emerged as a powerful tool in a variety of ecological applications, several considerations or sources of uncertainty remain when using the technique. The following

discussion will focus on those that are most relevant to the use of SIA in ecotoxicology studies and some of their associated caveats.

**Consideration/Source of Uncertainty 1: Unequal Diet-Tissue Stable Isotope Fractionation Among Species.** In order to quantify the biomagnification of a contaminant through a food web, the slope of the regression between, e.g., an organochlorine and the  $\delta^{15}\text{N}$  of the biota, is used as an overall descriptor and it is not necessary to assign a particular value to  $\Delta^{15}\text{N}$  (the difference in  $\delta^{15}\text{N}$  between an animal and its diet). We must simply agree that the diet–consumer fractionation approaches some consistent value when averaged over the entire food web and that the slope of the contaminant vs  $\delta^{15}\text{N}$  regression reflects biomagnification across the entire food web and not changing  $\Delta^{15}\text{N}$ . However, when converting  $\delta^{15}\text{N}$  to TL estimates (31), a value for  $\Delta^{15}\text{N}$  must be chosen. This is also necessary when deriving biomagnification factors (BMFs) within different compartments of the food web (40) to compare results with those of prior studies that did not use SIA (e.g., ref 69). BMFs are calculated by dividing the ratio of predator to prey contaminant concentrations by the ratio of their TLs (40).

Recently, the use of primary consumers over primary producers as a baseline of a particular food web has led to the modification of eq 4. TLs of organisms in the food web are now typically calculated according to

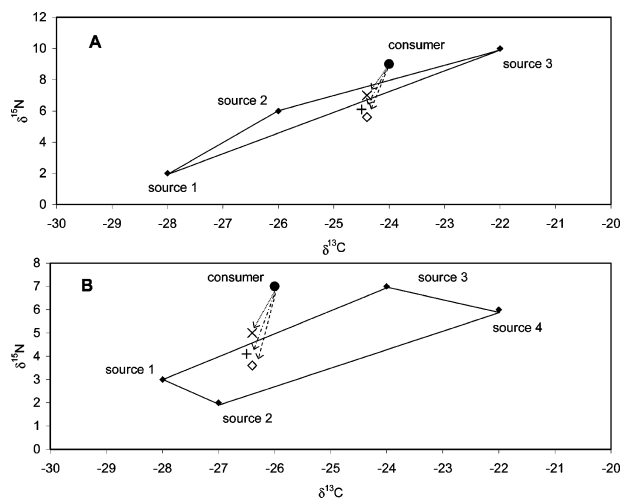
$$\text{TL}_{\text{organism}} = (\delta^{15}\text{N}_{\text{organism}} - \delta^{15}\text{N}_{\text{baseline}}) / \Delta^{15}\text{N} + 2 \quad (8)$$

where  $\delta^{15}\text{N}_{\text{baseline}}$  is the measured  $\delta^{15}\text{N}$  of a long-lived primary consumer (TL = 2). A value between 2 and 5‰ is often assigned to  $\Delta^{15}\text{N}$ , although this may vary depending on the types of organisms within the food web and is the subject of some debate (70). Bivalve mollusks such as mussels (4, 12, 35) and clams (71, 72) have been commonly used to standardize the baseline of food webs, but other taxa have been also been used including gastropods (4, 47), copepods (40, 54, 55, 73), and other invertebrates (12). When baseline  $\delta^{15}\text{N}$  varies between two habitats within an ecosystem, the trophic position model accounts for individuals foraging in these habitat zones (e.g., pelagic vs littoral zones of lakes) using

$$\text{TP} = 2 + \{ \delta^{15}\text{N}_{\text{organism}} - [\delta^{15}\text{N}_a \times P_a + \delta^{15}\text{N}_b \times (1 - P_a)] \} / \Delta^{15}\text{N} \quad (9)$$

where  $\delta^{15}\text{N}_a$  and  $\delta^{15}\text{N}_b$  are stable nitrogen ratios of primary consumers from habitats a and b, respectively.

The choice of  $\Delta^{15}\text{N}$  (and even  $\Delta^{13}\text{C}$ ) becomes important in these models because it affects calculations of the importance of different sources to an organism's diet. When using the overall mean  $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$  reported by multiple authors (4–6, 70, 74), calculations result in the consumer's mixture sometimes falling outside the mixing space used to estimate proportional contributions of different diet items (Figure 2, 62). This stems from spatial patterns in isotopic distributions and trophic fractionation, wherein  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  are often correlated (with a positive  $r$ , i.e., foods that are enriched in  $^{15}\text{N}$  are often also enriched in  $^{13}\text{C}$ , and both  $^{15}\text{N}$  and  $^{13}\text{C}$  increase to some degree up the food chain); this, in turn, generates obtuse triangles (Figure 2a) or laterally compressed polygons (Figure 2b) in dual isotope space (e.g., ref 75). This leaves little isotopic "space" for the mixture to fall within and to provide feasible data on relative contributions of different sources to an organism's diet. In this case, researchers are left with the dilemma of choosing some other value for  $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$ . For example, if the species of interest is northern pike (*Esox lucius*), one could choose literature estimates for  $\Delta^{15}\text{N}$  of 3.4‰ (4, 74), 2.0‰ (6), 2.9‰



**FIGURE 2.** Hypothetical consumer stable carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotopic ratios (solid circles), along with three (A) and four (B) potential food sources, and back-calculated dietary mixtures after correcting for diet–tissue  $^{13}\text{C}$  and  $^{15}\text{N}$  fractionation using values from ref 5 (+’s), ref 4 (open diamonds), and ref 6 (x’s). Note that in scenario A, feasible source contributions are obtained when using fractionation estimates from ref 6, while in scenario B, using the same fractionation estimates (ref 6) places the mixture outside of feasible source contributions.

**TABLE 1.** Possible Choices for Diet–tissue  $^{15}\text{N}$  Fractionation ( $\Delta^{15}\text{N}$ ) for Carnivorous Fishes When Constructing Food Webs Without a *priori* Knowledge of  $\Delta^{15}\text{N}$  from Captive Studies

grouping	value (‰)	reference
all taxa	3.4	74
	2.9	5
	3.4	4
	2.0	6
	2.5	70
carnivores	3.2	5
	2.9	6
	2.7	70
organisms that excrete ammonia	2.3	6
	2.0	70

(5), or 2.5‰ (70) (Table 1). These are pooled estimates for all animals, on all types of diets, and from a number of different environments. Within each of these studies attempts have been made to account for differences in  $\Delta^{15}\text{N}$  based on taxonomy, method of nitrogenous waste excretion, etc., with the hope that this could provide a more accurate value of  $\Delta^{15}\text{N}$  for the animal of interest. However, the range of values for  $\Delta^{15}\text{N}$  in the literature still leaves a difficult decision for the researcher studying northern pike, which happens to be a carnivorous fish that excretes ammonia. Should the value of 2.7‰ for carnivores or 2.0‰ for animals that excrete ammonia be used (70)? The only true solution in this case is to rear northern pike on a known diet and then calculate  $\Delta^{15}\text{N}$  directly, but this could be a costly and labor-intensive exercise, and it may take years to achieve isotopic equilibrium of the fish with its diet (76).

It has been argued that this assumption is less of a concern when using  $\delta^{15}\text{N}$  to measure biomagnification because variable values of  $\Delta^{15}\text{N}$  will be averaged out over multiple TLs and approach 3.4‰, especially when primary consumers are used as the baseline (5). There is far greater  $\Delta^{15}\text{N}$  variability in herbivores compared to carnivores (5); therefore, by eliminating the highly variable initial link between primary producers and primary consumers by using shellfish or another obligate herbivore as a baseline in biomagnification

studies (73), our confidence in 3.4‰ as a value for  $\Delta 15N$  increases. As well, 3.4‰ as a value for  $\Delta 15N$  becomes more plausible with the inclusion of vertebrate species, which tend to be large, long-lived, and less vulnerable to rapid isotopic turnover as compared to invertebrates. For example, strong relationships between contaminant concentrations and TL or  $\delta^{15}N$  were found for a food web that included zooplankton and vertebrates (40), but when the contaminant-TL relationships only considered zooplankton the relationships were not as strong or were not significant (73). This may be due in part to issues such as body size and habitat that may be more important in determining contaminant concentrations in zooplankton than their feeding ecology (77, 78).

**Consideration/Source of Uncertainty 2. Variable Diet-Tissue Stable Isotope Fractionation Within a Species.** In the example above, if the researcher had done a captive rearing experiment to determine  $\Delta 15N$  and  $\Delta 13C$  for northern pike, a further assumption is required before the results are applied to field studies. It must be assumed that the  $\Delta 15N$  values are static and not affected by environmental conditions, such as temperature, that would fluctuate over seasons for wild animals. Experimental evidence suggests that this assumption may be invalid. Changes in isotopic signatures of *Daphnia magna* on a fixed diet were related to differences in temperature across tanks (79). Changes in walleye  $\delta^{15}N$  were independent of dietary switches or growth but were significantly related to age (80), suggesting that animals become progressively enriched in  $^{15}N$  over time despite always feeding at the same TL. This could confound interpretation of TL estimates for older consumers. However, no relationship between age and  $\delta^{15}N$  was found for lake trout (49), and size, as a surrogate for TL, is a better predictor of  $\delta^{15}N$  than age among rainbow smelt (*Osmerus mordax*) morphotypes (81). These latter observations support a relatively consistent enrichment factor between an animal and its diet over the animal's lifetime.

Previous studies have demonstrated that the nutritional state of the animal can have a strong influence on  $\Delta 15N$  (82–84). During starvation or other periods of high nitrogen demand, excretion of concentrated nitrogenous waste products (e.g., urea) that are isotopically “light” (85) leads to  $^{15}N$  enrichment in the remaining nitrogen pool available for anabolism. Animals that are stressed have been shown to have higher  $\delta^{15}N$  than their non-stressed counterparts, and certain tissues appear to be more affected by this stress than others (82–84). The mechanism underlying this phenomenon is relatively unknown but is likely related to  $^{15}N$  fractionation during the transfer of nitrogen (transamination and deamination) between amino acids (86, 87) in elimination processes. The relationships between nutritional state and  $\delta^{15}N$  have led to investigations into the effect of dietary quality on  $\Delta 15N$ . While theoretically plausible, results to date have been equivocal, with some authors finding strong associations between diet quality and  $\Delta 15N$  (88, 89), and others finding weak or variable relationships (70, 90, 91).

Another complication to the application and use of  $\Delta 13C$  values is the process of endogenous lipid synthesis, which can affect the  $\Delta 13C$  between an animal and its diet if the consumer has a higher proportion of lipids when compared to its prey. Lipids are isotopically lighter due to fractionation against the heavier isotope during the oxidation of pyruvate to acetyl-CoA during lipid formation (92). Some studies have used lipid-extracted tissues to remove the effects of this confounding variable (e.g., ref 42); lipids are removed with a solvent such as chloroform-methanol (93). However, lipid extraction has been shown to influence  $\delta^{15}N$  (94), possibly due to protein degradation during the extraction process. In addition, animals that are reared on a constant diet but have different body compositions or metabolic characteristics can show variations in  $\Delta 13C$  of up to 1.5‰ in lipid-free matter,

suggesting that lipid load is not solely responsible for more negative  $\delta^{13}C$  in the predator (95, 96). An alternative to lipid extraction is lipid normalization using carbon to nitrogen ratios (C:N) that are often provided by analytical labs alongside stable isotope data. Percent lipid can be related to C:N in tissues, as for a marine food web (97), and the  $\delta^{13}C$  values can be adjusted accordingly. A similar approach can be used to normalize contaminant concentrations (98) without having to lipid-extract tissues for SIA.

The lipid issue is particularly relevant to ecotoxicological studies on lipophilic contaminants, and is confounded by the fact that lipids are highly correlated to both trophic position and concentrations of these pollutants (e.g., ref 50). For this reason, many studies express concentrations of organic pollutants on a lipid weight basis, thus accounting for increased % lipid up the food chain, and then use  $\delta^{15}N$  to describe the remaining variability in contaminant concentrations among species or individuals (e.g., refs 40, 50).

**Consideration/Source of Uncertainty 3. Different Tissues from an Individual Animal Have Variable Stable Isotope Ratios.** If assumptions 1 and 2 are met, i.e.,  $\Delta 15N$  and  $\Delta 13C$  have been established and it is unlikely that the nutritional state of the animal is a concern under the experimental conditions, the researcher must then choose an appropriate tissue to represent the animal's diet. However, because different tissues may have different degrees of fractionation relative to the diet (88) and turnover rates (76, 99), a single tissue may not be adequate in tracing the flow of organic matter and contaminants through an ecosystem (100). These two issues (fractionation and turnover) will be examined separately.

**Different Fractionation Among Tissues.** When animals are on a fixed diet, and hence are in isotopic equilibrium, isotopic ratios may vary among tissues due to their differential composition of proteins, lipids, and carbohydrates (88). For example, variation (up to 4‰) in isotope ratios among tissues (whole body, muscle, heart and liver) has been reported for lab-reared rainbow trout (*Oncorhynchus mykiss*, 101), while different diet-tissue fractionations of  $^{13}C$  and  $^{15}N$  in blood, liver, muscle, collagen, and feather have been shown in several bird species (88). As discussed previously for among-individual comparisons, the lipid load of a specific tissue may also affect inter-tissue differences in  $\delta^{13}C$ . Lipid rich tissues such as liver and eggs tend to have lower  $\delta^{13}C$  than low-lipid tissues such as muscle, hair, and feathers (84, 102). Differences in amino acid composition may also determine the distribution of heavy and light carbon and nitrogen isotopes in different tissues, as certain amino acids have been shown to be “heavier” than others (103).

**Different Turnover Rates Among Tissues.** When animals switch diets to one with a unique isotopic ratio, the rate of isotopic change toward that of the new diet also varies among tissues. Different rates of turnover among tissues was first shown in gerbils (*Meriones unguiculatus*), with faster turnover in liver (half-life = 6.4 days) compared to muscle (half-life = 27.6 days) and hair (half-life = 47.5 days) (102). These differences were related to differential metabolic activity of the tissues. Different tissues from animals that periodically switch diets in response to changes in habitat or prey availability may have distinctive isotopic ratios depending on the turnover rate of the tissue, the duration of the diet switch, and the isotopic composition of the new diet.

For these reasons, analysis of several tissues for both stable isotopes and contaminants may offer additional information on the temporal dynamics of contaminant uptake than would be available from a single tissue (Table 2). Other factors to consider when choosing tissues include the ability to sample without sacrificing the animal (104, 105) and the mass of the tissue required for SIA and contaminant analyses. Following earlier recommendations (101), most researchers have settled

**TABLE 2. Features of Different Tissues Used for Stable Isotope Analysis in Ecotoxicology Studies<sup>a</sup>**

tissue	turnover rate	non-lethal sampling?	heterogeneity	other issues
whole body	intermediate	no	high	
white muscle	intermediate	no <sup>b</sup>	low	difficult to remove from small individuals
liver	rapid	no	high	
fin	intermediate	yes	unknown	small tissue volume; other measurements impossible
feather	slow	yes	high	accumulate heavy metals
hair	slow	yes	high	
whole blood	intermediate	yes	low	
blood plasma	rapid	yes	low	small tissue volume; other measurements unlikely
blood cells	intermediate	yes	low	small tissue volume; other measurements unlikely

<sup>a</sup> Heterogeneity refers to potential within-tissue variance (e.g., base to tip of a feather). <sup>b</sup> Non-lethal "muscle plugs" possible in larger fish.

on muscle tissue as the logical choice for fishes in food web studies because of its low lipid content, intermediate turnover rate, and relevance to fish consumption by humans; however, the liver, which has been shown to exhibit a faster turnover rate in fishes (76, 106, 107), may also be important in ecotoxicology studies as a center for metabolic clearance of contaminants. In birds and mammals, liver turns over more rapidly than other body tissues (99, 102), and therefore, could be used to assess shorter-term dietary changes.

**Consideration/Source of Uncertainty 4. Baseline Stable Isotope Ratios Vary Across Systems.** Inputs of nutrients from exogenous sources, both natural and anthropogenic in origin, are common in many aquatic food webs (108, 109). These nutrients often have distinct isotopic ratios (e.g., ref 110), their influence can vary spatially and temporally (111), and as a result can cause primary producers to vary in their isotopic ratios both within and across systems. Changes in baseline isotope ratios can be due to anthropogenic influences (35) such as human sewage and agriculture (112), or natural nitrogen transformations, including nitrification and denitrification (71). Fluctuating baseline  $\delta^{13}\text{C}$  and especially  $\delta^{15}\text{N}$  have the potential to confound interpretation of trophic differences within a species when one compares across systems or over a spatial gradient within a system. For example, despite a relatively low variance in baseline  $\delta^{15}\text{N}$  among three lakes, sensitivity analysis revealed large potential effects of these different baselines on TL estimates for higher order consumers (4). Far greater effects of varying baselines are expected when comparing organisms from areas of dense human activity with more pristine locations (35).

**Consideration/Source of Uncertainty 5. The Presence of True Omnivores.** While debate continues over the influence of omnivory (feeding on more than one TL) on the stability of food webs and its ubiquity in general (113, 114), field studies suggest that there are indeed instances where omnivory does occur and stable isotope data have supported this conclusion (34, 115). It is important to consider omnivory in ecotoxicology studies that use SIA because periodic omnivory may lead to a disproportionate uptake of contaminants when compared to any concurrent shifts in isotopic ratios. This may be a consequence of differences in the kinetics of the uptake and excretion of organic matter and contaminants. Bioenergetics models for certain contaminants (e.g., methylmercury and cesium) predict that fish with high activity and food consumption rates will more rapidly accumulate contaminants than they will accumulate biomass (116). Since isotopic change following a diet switch in ectotherms is thought to be dominated by growth (117, 118, but see ref 76), it follows that a switch in diet by this organism to a prey type with a large contaminant load might generate higher contaminant concentrations for the consumer than predicted based on isotopic ratios alone (119). Likewise for endotherms, half-lives of many contaminants are far greater than the turnover rate of carbon and nitrogen in tissue proteins (120–

122), resulting in higher-than-expected contaminant burdens for, e.g., birds that scavenge marine mammals with large contaminant loads (42, 123). In this type of application, contaminant information can in fact aid in the interpretation of stable isotope data.

**Consideration/Source of Uncertainty 6. Movement of Animals and Nutrients Between Food Webs/Ecosystems.** Despite the integrative nature of SIA, one-time collection and analysis of food web components (unless different tissues with different turnover rates are used) essentially presents a static measure of food web structure and energy and contaminant flow. This may fail to represent the dynamic nature of temporally variable food webs, particularly those that receive periodic influxes of nutrients, organic matter, and contaminants from adjacent systems (108). Certain migratory species may accumulate large body burdens of contaminants that can then be deposited in breeding habitats through excretion, predation, and decomposition (124). High methylmercury concentrations in invertebrates inhabiting a Lake Ontario tributary following a large run of spawning Chinook salmon (*Oncorhynchus tshawytscha*) has been demonstrated (125). Differences in organochlorine residues among populations of grizzly bears (*Ursus arctos horribilis*) in British Columbia, Canada were related to the seasonal consumption of a more contaminated diet of salmon by one population; this difference in dietary habits was determined using hair isotopic ratios (126). Coastal dwelling bears that shifted diets to salmon had higher concentrations of  $\Sigma\text{DDT}$ ,  $\Sigma\text{CHL}$ , and dieldrin than their counterparts that were almost exclusively herbivorous (126).

A second challenge in this approach arises from the presence of migratory species that arrive from habitats with higher or lower baseline contaminant concentrations or isotope ratios; these individuals, therefore, appear as outliers in contaminant vs  $\delta^{15}\text{N}$  plots or in dual isotope plots. For example, higher-than-expected concentrations of persistent organic pollutants in black-legged kittiwakes (*Rissa tridactyla*) in the Canadian Arctic were presumed to stem from the accumulation of these pollutants from their wintering grounds on the eastern North American seaboard (40). Similarly, residents and (tributary) migrants in a population of American dippers (*Cinclus mexicanus*), identified using SIA, had significantly different contaminant concentrations (127). Resident dippers ate significantly more fish than the migrants, leading to higher Hg, organochlorines, and PCBs in the former cohort (127).

Perhaps the best examples of complex systems with constant species movements and multiple food sources are estuaries (128). Species found in the estuary at any particular time may spend significant time outside the estuary in freshwater or marine water where contaminants and stable isotope ratios likely differ. Interestingly, there have been few published studies that have examined food web behavior of contaminants in estuaries, which may be due in large part

to the complexity of these systems. For example, few significant relationships between PCB congener concentrations and TL were found for a Georgia, U.S., estuarine food web, despite high PCB concentrations in the biota (129). Likewise, only weak relationships between mercury and TL were found for an estuarine food web in New Brunswick, Canada (130). Understanding nutrient and organic matter exchange within estuaries may, therefore, be important in assessing contaminant transfer on a larger scale (108).

**New Directions and Recommendations.** Over the past few years, SIA has been used in new ways to study ecotoxicology. Because contaminant concentrations in biota are affected by the complex interplay between several environmental processes, researchers have begun to use isotopic ratios in conjunction with physical, chemical, or biological factors to generate more sophisticated models to understand contaminant concentrations in consumers (53, 131, 132). A comprehensive analysis of biomagnification slopes from contaminant vs  $\delta^{15}\text{N}$  associations available in the literature (37, 133) may prove useful in understanding factors, such as contaminant properties, food web structure, and productivity that lead to higher-than-normal rates of biomagnification in aquatic systems.

The effect of invasive species on contaminant concentrations can also be assessed using SIA (31, 34). One such species, rainbow smelt, has been shown to lengthen food chains (31, 72). However, while smelt invasion resulted in higher Hg concentrations in lake trout in boreal lakes (31), similar Hg concentrations were found in the top predator walleye (*Sander vitreus*) in both smelt-invaded and reference lakes (72), suggesting that other factors, such as ecosystem productivity, are interacting with trophic transfer to control Hg biomagnification (134).

Assessing toxicological effects and dietary habits concurrently will improve our ability to determine the ecological relevance of changes in food pathways for those contaminants for which dietary exposure is significant. For example, marine consumers connected to a clam-based food web accumulated selenium more rapidly through the food web (higher selenium- $\delta^{15}\text{N}$  slopes) than those animals in a food web supported by crustaceans within the same region (135). This steep biomagnification slope in the clam-based food web pushed several species above the toxicity threshold for selenium (135). In a lab trial, snowy egrets (*Egretta thula*) exposed to high levels of methylmercury had tissue-specific (muscle and liver) shifts in isotopic ratios that were likely associated with protein stress and degradation (136).

Analysis of archived tissues could provide information on spatial and temporal trends in contaminant concentrations (e.g., museum collections, 137). These time series will allow us to determine if environmental concentrations are increasing or decreasing, particularly in relation to bureaucratic decisions that regulated releases, such as the banning of DDT in North America (20) or the introduction of the Clean Air Act in the United States. Pairing the archived contaminant data with stable isotope analyses of the same tissues (138), provided baseline  $\delta^{15}\text{N}$  has not changed, may allow researchers to account for long-term changes in trophic structuring that could alter contaminant profiles in consumers (139).

The use of compound-specific stable isotope analyses, both in the tissue fraction of consumers (140) and in the contaminants themselves (141), is gaining popularity and will no doubt add to our understanding of contaminant cycling within organisms and through food webs, and to our ability to link contaminants in consumers to their original source.

SIA has had an almost exponential increase in its use in ecology and ecotoxicology studies over the past several decades (142), and its number of applications will no doubt

continue to expand. In order to improve interpretation of stable isotope data, we recommend the following:

When using SIA across multiple systems in ecotoxicology studies, it is critical to standardize the  $\delta^{15}\text{N}$  of an organism to the basal value within each system to avoid inappropriate conclusions with respect to relative TL. Typical variation in baseline  $\delta^{15}\text{N}$  among ecosystems (range of  $\sim 12\text{‰}$ , ref 35) outlined in consideration 4 outstrips the variability associated with considerations 1–3: (1) interspecific differences in  $\Delta 15\text{N}$  (range of  $\sim 5\text{‰}$ , ref 4), (2) intraspecific differences in  $\Delta 15\text{N}$  associated with the physiology of the animal (range of  $\sim 5\text{‰}$ , refs 82, 89), or (3) variability among tissues within an animal (range of  $\sim 5\text{‰}$ , refs 3, 70, 101). Indeed, considerations 2 and 3 are embedded within consideration 1, in that much of the variability among species is likely related to differences in physiology and tissues sampled (70).

Proper procedures for assessing baselines in lakes has been outlined (4), and it is recommended to use SIA of filter feeding mussels to quantify the pelagic baseline and snails that feed on benthic algae to establish the littoral baseline. This was an advance from the use of zooplankton that can have considerable interspecific or seasonal variation in isotopic ratios within lakes (143). Unfortunately, proper techniques for baseline assessment in small fluvial systems are less well developed, in part due to the rarity of long-lived primary consumers in these habitats (but see ref 144). Further development of baseline assessment, perhaps using a variety of functional feeding groups (e.g., scrapers and shredders, 145), would be useful for future stable isotope and ecotoxicology studies in streams and rivers.

In order to assess species-specific diet-tissue fractionation, study animals should be reared on a constant diet in a laboratory setting. If this is not feasible, use available information from the literature and choose the most appropriate values for  $\Delta 15\text{N}$  and  $\Delta 13\text{C}$  (4–6, 70, 74).

Whenever possible, incorporate other biological information to the study, particularly animal movement patterns and short-term shifts in diet (i.e., omnivory). Supplementing of isotope data with more traditional measures of feeding behavior, such as gut content analysis (GCA) and visual observations, as well as analysis of multiple tissues for SIA, will enhance understanding of contaminant data that may not have been revealed with isotope analysis of a single tissue (119). For the most part, past studies have found relatively good agreement between GCA and SIA (146, 147).

By considering the sources of uncertainty outlined in this paper and by continuing to conduct controlled laboratory studies that better define isotope kinetics, we hope that future research with SIA will continue to provide new insights into factors that govern the movement of organic matter and contaminants through ecosystems.

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