

Combined use of radiocarbon and stable carbon isotopes for the source mixing model in a stream food web

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Abstract

Radiocarbon natural abundance ($\Delta^{14}\text{C}$) has emerged as a useful dietary tracer in freshwater ecology for the past decade, yet its applicability for separating aquatic and terrestrial resources has not been examined quantitatively. Here, we report $\Delta^{14}\text{C}$ values of stream invertebrates in different functional feeding groups collected from the upper South Fork Eel River watershed, northern California. We found that algae-grazing insect larvae show low $\Delta^{14}\text{C}$ values ($-43.1 \pm 21.8\text{‰}$, mean $\pm$ standard deviation, $N = 6$), reflecting the signal of dissolved inorganic carbon weathered from ancient inorganic carbon or respiration of old organic carbon. In contrast, the $\Delta^{14}\text{C}$ values of leaf-shredding insect larvae ($21.7 \pm 31.9\text{‰}$, $N = 5$) were close to those of contemporary atmospheric CO_2 except at the SF Eel River where algal production was highest. The $\Delta^{14}\text{C}$ values of predators ($-6.1 \pm 35.7\text{‰}$, $N = 14$) were intermediate between those of grazers and shredders. In a Bayesian mixing model, $\Delta^{14}\text{C}$ provided a more ecologically realistic estimate for terrestrial vs. aquatic source contributions to invertebrates with lower uncertainty (i.e., narrower credible interval) than did the stable carbon isotopes ($\delta^{13}\text{C}$). These results demonstrate that $\Delta^{14}\text{C}$ can be used, in combination with $\delta^{13}\text{C}$, to more precisely estimate organic matter sources to stream animals.

Stream ecosystems are supported by both aquatic (e.g., benthic algae, aquatic macrophytes, or phytoplankton) and terrestrial (e.g., leaf litter) primary production. Understanding the relative importance of different carbon sources for food webs has been one of the most fundamental challenges in stream ecology for the past 40 yr (Vannote et al. 1980). Stable carbon isotopes ($\delta^{13}\text{C}$) have been useful as diet tracer in stream food web studies when aquatic and terrestrial resources can be separated using $\delta^{13}\text{C}$ values (Finlay 2001). In addition to $\delta^{13}\text{C}$, several other isotope tracers, such as hydrogen (Doucett et al. 2007), mercury (Tsui et al. 2014), and magnesium (Nitzsche et al. 2019), have been used for tracing material flow at the land–water interface. Radiocarbon natural abundances

($\Delta^{14}\text{C}$) have also drawn attention for estimating carbon sources to stream food webs (e.g., Caraco et al. 2010; Ishikawa et al. 2014; Fellman et al. 2015; Bellamy et al. 2017).

The applicability of $\Delta^{14}\text{C}$ for separating aquatic and terrestrial resources in streams depends on the source of dissolved inorganic carbon (DIC). DIC is used by aquatic photoautotrophs such as algae or macrophytes, and in turn is passed on through food chains to higher trophic level such as invertebrates and fish. If DIC is completely equilibrated with atmospheric CO_2 , the $\Delta^{14}\text{C}$ method does not work in stream food web studies because the $\Delta^{14}\text{C}$ values of aquatic and terrestrial resources would fall into the same (modern) range (Bellamy and Bauer 2017). However, stream water DIC is derived not only from atmospheric CO_2 , but also from weathered carbonates, shales, and degraded organic matter, all of which show lower $\Delta^{14}\text{C}$ values than the contemporary atmospheric CO_2 (Fellman et al. 2015; Ishikawa et al. 2015a). Currently, the applicability of $\Delta^{14}\text{C}$ is poorly known particularly for stream food webs in watersheds lacking extensive carbonate mineral bedrock (e.g., limestone), which is one of the most significant sources of ^{14}C -dead (i.e., $\Delta^{14}\text{C} = -1000\text{‰}$) inorganic carbon into the

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stream water DIC (Ishikawa et al. 2014). Therefore, testing stream food webs without limestone bedrock is key to demonstrating the potential of $\Delta^{14}\text{C}$ for the aquatic vs. terrestrial source separation.

We conducted this study in the upper South Fork Eel River watershed, northern California (see Power 1990; Finlay 2001; Uno and Power 2015 for site descriptions). The geology of the region is Early Tertiary Complex with little or no limestone (McLaughlin et al. 2000). It should be noted that marine shales and mudstones in bedrock of the Eel River (Hahm et al. 2019) likely have some carbonate minerals (Finlay 2003) that could influence stream water $\Delta^{14}\text{C}$ (Blair et al. 2003; Leithold et al. 2005). We hypothesized that the aquatic and terrestrial resources are still distinguishable without the presence of limestone, because the organic matter mineralization in water column also provides ^{14}C -depleted carbon to DIC and aquatic resources such as periphytic algae (hereafter periphyton) (Ishikawa et al. 2015a). To test the above hypothesis and systematically compare $\Delta^{14}\text{C}$ with $\delta^{13}\text{C}$, the most widely used carbon tracer, we collected periphyton, leaf litter, and benthic invertebrates from five tributary streams in the Eel River. The $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ values of organisms were measured to estimate relative contributions of aquatic and terrestrial resources to the Eel River food web. A Bayesian mixing

model was employed to examine models with which isotopes show the highest performance, $\delta^{13}\text{C}$, $\Delta^{14}\text{C}$, or both.

Materials and methods

Study sites and sample collection

We collected samples from five study sites in the South Fork (SF) Eel River in the Angelo Coast Range Reserve (39°44'17"N, 123°37'48"W) (Fig. 1) in July 2013. The five streams consist of McKinley Creek (watershed area 1.0 km²), Skunk Creek (1.4 km²), Fox Creek (2.6 km²), Elder Creek (17.0 km²), and SF Eel River (main stem, 130.0 km²). Stream water CO₂ concentrations varied among the five study sites, ranging from very high levels in small streams (> 300 $\mu\text{mol L}^{-1}$) to undersaturated levels during daytime in sunny, productive river reaches (< 10 $\mu\text{mol L}^{-1}$) (Finlay 2003). Macroinvertebrate larvae with different functional feeding groups, such as grazers (*Glossosoma penitum*, N = 3; *Neophylax splendens* and *Neophylax rickeri*, N = 1; or *Dicosmoecus gilvipes*, N = 2); shredders (*Lepidostoma* spp., N = 4; or *Psychoglypha bella* and *Psychoglypha leechi*, N = 1); and predators (*Calineuria californica*, N = 14), were collected from streambed by hand. Their potential carbon sources, including periphyton, leaf litter, an aquatic moss (*Fontinalis antipyretica*, N = 2), and filamentous algae (*Cladophora glomerata*, N = 3), were

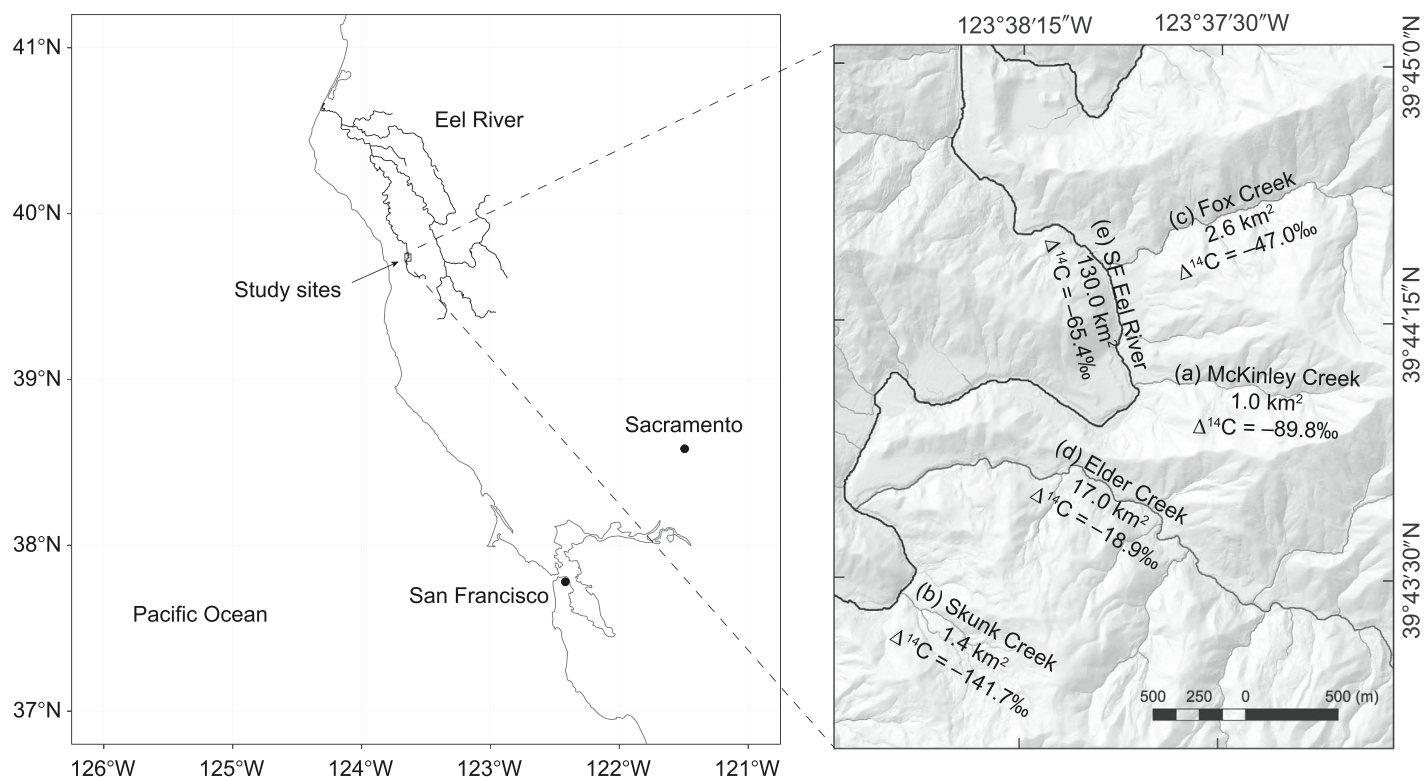


Fig. 1. Map of study sites in the Angelo Coast Range Reserve in the Eel River watershed, northern California. The $\Delta^{14}\text{C}$ values of periphyton are shown as the end-member of aquatic carbon source for each of the five studied streams.

collected using a brush or by hand from several submerged cobles or from stream bed, and were washed using stream water before transferred into the laboratory using precombusted (450°C, 5 h) glass vials. Water striders (*Gerris remigis*, predators at the land–water interface, $N = 5$) were also collected from the stream surface by hand. All invertebrate samples were identified visually, by eye or under a microscope. For very small grazers and shredders, several individuals belonging at least to the same genus were batched for isotopic analysis. The samples of periphyton, *F. antipyretica*, and *Cladophora glomerata* were washed using 0.1 mol L⁻¹ HCl to remove possible carbonate contamination. All samples were dried using oven at 60°C for overnight, and homogenized to fine powder before isotope analysis.

Isotope measurements

We measured the stable carbon isotope ratios of samples using an elemental analyzer connected to an isotope ratio mass spectrometer via a ConFlo III interface (Thermo Fisher Scientific) (Ogawa et al. 2010). The isotope ratios were expressed as conventional $\delta^{13}\text{C}$ values against the international standard of Vienna Pee Dee Belemnite (VPDB):

$$\delta^{13}\text{C} (\text{‰}) = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 10^3$$

$$R = \frac{^{13}\text{C}}{^{12}\text{C}} \quad (1)$$

The analytical precision of the $\delta^{13}\text{C}$ measurements was within 0.2‰.

Radiocarbon concentration was measured after graphitization following the method described in Ishikawa et al. (2014). Briefly, the powdered sample was combusted in an evacuated quartz tube with copper, copper oxide, and silver at 500°C for 30 min and 850°C for 2 h. The generated CO₂ was cryogenically purified in a vacuum line, and reduced to graphite using hydrogen and an iron catalyst at 650°C for 6 h. The $\Delta^{14}\text{C}$ values of the graphite samples were determined using accelerator mass spectrometry (AMS) at the Institute of Accelerator Analysis (Kawasaki, Japan). The radiocarbon content was reported as $\Delta^{14}\text{C}$ value after the $\delta^{13}\text{C}$ correction as follows (Stuiver and Polach 1977):

$$\Delta^{14}\text{C} (\text{‰}) = \delta^{14}\text{C} - 2 (\delta^{13}\text{C} + 25) \left(1 + \frac{\delta^{14}\text{C}}{1000} \right) \quad (2)$$

where $\delta^{14}\text{C}$ is defined as ^{14}C activity of sample relative to the international standard (Hox II oxalic acid). The analytical error was within 3‰. The apparent radiocarbon age (i.e., Libby age; year before present: yr B.P.; A.D. 1950 = 0 yr B.P.) was derived using the $\Delta^{14}\text{C}$ value as follows (Stuiver and Polach 1977):

$$\text{Radiocarbon age (yr B.P.)} = -8033 \ln \left(\frac{\Delta^{14}\text{C}}{1000} + 1 \right) - (\gamma - 1950). \quad (3)$$

where γ is the year when the radiocarbon measurement was conducted (either 2014 or 2019).

Mixing model

We estimated relative contributions of periphyton and leaf litter to invertebrates among (1) $\delta^{13}\text{C}$ -based, (2) $\Delta^{14}\text{C}$ -based, and (3) $\delta^{13}\text{C}$ - $\Delta^{14}\text{C}$ -combined mixing models. A Bayesian mixing model (Stable Isotope Analysis in R; SIAR) 4.1 (Parnell et al. 2010) based on either (1), (2), or (3) was used with minimal prior information (i.e., the Dirichlet prior distribution assuming that all food sources are equally probable). The Markov Chain Monte Carlo simulations were carried out with 1,000,000 iterations, 400,000 burn-ins, and 300 thinnings. The mean and standard deviation (SD) of $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ values of periphyton and leaf litter in five sites were combined, and used as the respective aquatic and terrestrial end-members for all invertebrates. This design was adopted because the aquatic end-member exhibited very large variations in both $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ among sites. The minor influence of trophic enrichment of ^{13}C was ignored (Vander Zanden and Rasmussen 2001) because any single enrichment factor could not be assigned a priori to consumers potentially belonging to different trophic levels including primary consumers (grazers and shredders) and secondary consumers (predators and water striders). Performance was compared among the above three models based on accuracy of the estimate (i.e., consistency with diet preference as predicted from independent evidence based on the gut content analysis in a previous study; McNeely et al. 2006) and precision of the estimate (i.e., the width of the posterior probability distributions). All statistics and graphics were carried out using R. 3.5.3. (R Core Team 2019).

Results

The $\delta^{13}\text{C}$ values of periphyton (i.e., aquatic source, $\delta^{13}\text{C} = -27.6 \pm 5.9\text{‰}$, mean $\pm$ SD, $N = 5$) and leaf litter (i.e., terrestrial source, $\delta^{13}\text{C} = -30.1 \pm 0.6\text{‰}$, $N = 5$) partially overlapped in the Eel River (combining data from all sites) due to the influence of water velocity on algal $\delta^{13}\text{C}$ (Finlay et al. 1999). In McKinley, Fox, and Skunk Creeks with watershed areas < 3 km², the $\delta^{13}\text{C}$ values of algae-grazers (*Glossosoma penitum*, *N. splendens*, and *N. rickeri*, -36.4‰ to -33.7‰) were $\sim 20\text{‰}$ lower than those (*Glossosoma penitum* and *D. gilvipes*, -17.4‰ to -14.1‰) in Elder Creek, and the SF Eel River (Fig. 2), and other larger tributaries in the Eel River (Finlay 2001, 2004). Leaf shredder $\delta^{13}\text{C}$ values were relatively constant across sites (*Lepidostoma* spp. and *Psychoglypha* spp., -23.1‰ to -20.1‰), except for shredders from the SF Eel River (*Lepidostoma* spp., -15.2‰), all of which were > 7‰ higher than their expected food source (i.e., leaf litter) $\delta^{13}\text{C}$ values. Predators and water striders showed variable $\delta^{13}\text{C}$ values (*Calineuria californica* and *Gerris remigis*, -27.5‰ to -18.9‰).

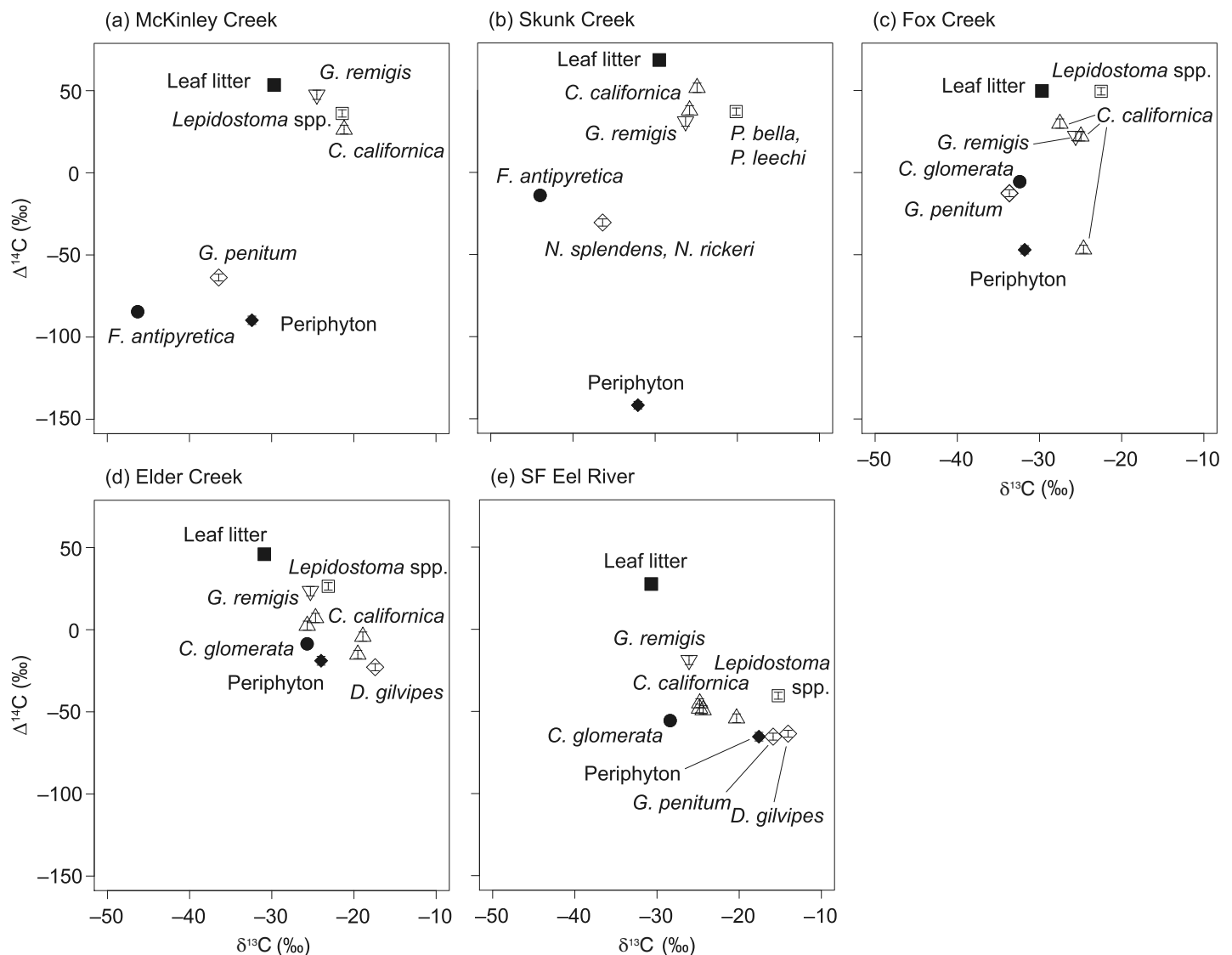


Fig. 2. $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ plots for periphyton (shaded diamond), green algae (shaded *Cladophora glomerata*, circle), an aquatic moss (the bryophyte *F. antipyretica*, shaded circle), leaf litter (shaded square), grazers (*Glossosoma penitum*, *N. splendens* and *N. rickeri* or *D. gilvipes*; open diamond); shredders (*Lepidostoma* spp. or *P. bella* and *P. leechi*; open square); predators (*Calineuria californica*; open triangle); and water striders (*Gerris remigis*; open reverse triangle) in (a) McKinley Creek, (b) Skunk Creek, (c) Fox Creek, (d) Elder Creek, and (e) SF Eel River. For $\Delta^{14}\text{C}$, AMS analytical errors are also shown.

The low $\Delta^{14}\text{C}$ values of periphyton ($\Delta^{14}\text{C} = -72.6 \pm 41.6\text{‰}$, $N = 5$) and the high $\Delta^{14}\text{C}$ values of leaf litter ($\Delta^{14}\text{C} = 49.0 \pm 13.2\text{‰}$, $N = 5$) were clearly discriminated in the Eel River (combining data from all sites). Grazers showed 31‰ on average lower $\Delta^{14}\text{C}$ values ($-43.1 \pm 21.8\text{‰}$, $N = 6$) than those of the other functional feeding groups (shredders: $21.7 \pm 31.9\text{‰}$, $N = 5$; predators: $-6.1 \pm 35.7\text{‰}$, $N = 14$; and water striders: $21.1 \pm 21.9\text{‰}$, $N = 5$). The $\Delta^{14}\text{C}$ values of all invertebrates were intermediate between those of periphyton and leaf litter (Fig. 2). The apparent radiocarbon age for grazers in all sites ($N = 5$) ranged from 38 to 479 yr B.P. Other invertebrates showed modern radiocarbon age except for predators *Calineuria californica* in Fox Creek ($N = 1$, 315 yr B.P.) and

Elder Creek ($N = 1$, 53 yr B.P.) and water striders *Gerris remigis* ($N = 1$, 83 yr B.P.), shredders (*Lepidostoma* spp., $N = 1$, 268 yr B.P.), and predators *Calineuria californica* ($N = 4$, 301–378 yr B.P.) in the SF Eel River site.

The SIAR model simulation using $\delta^{13}\text{C}$ -only, $\Delta^{14}\text{C}$ -only, and combined $\delta^{13}\text{C}$ - $\Delta^{14}\text{C}$ returned different estimates of periphyton and leaf litter contributions to the studied invertebrates (Fig. 3). When $\Delta^{14}\text{C}$ was used in the model, both the contribution of periphyton to grazers (mode: 68%; mean: 70%, Fig. 3a) and contribution of leaf litter to shredders (mode: 68%; mean: 67%, Fig. 3b) were high, which is consistent with their respective trophic ecology. A high contribution of periphyton to predators (mode: 94%; mean: 77%) was

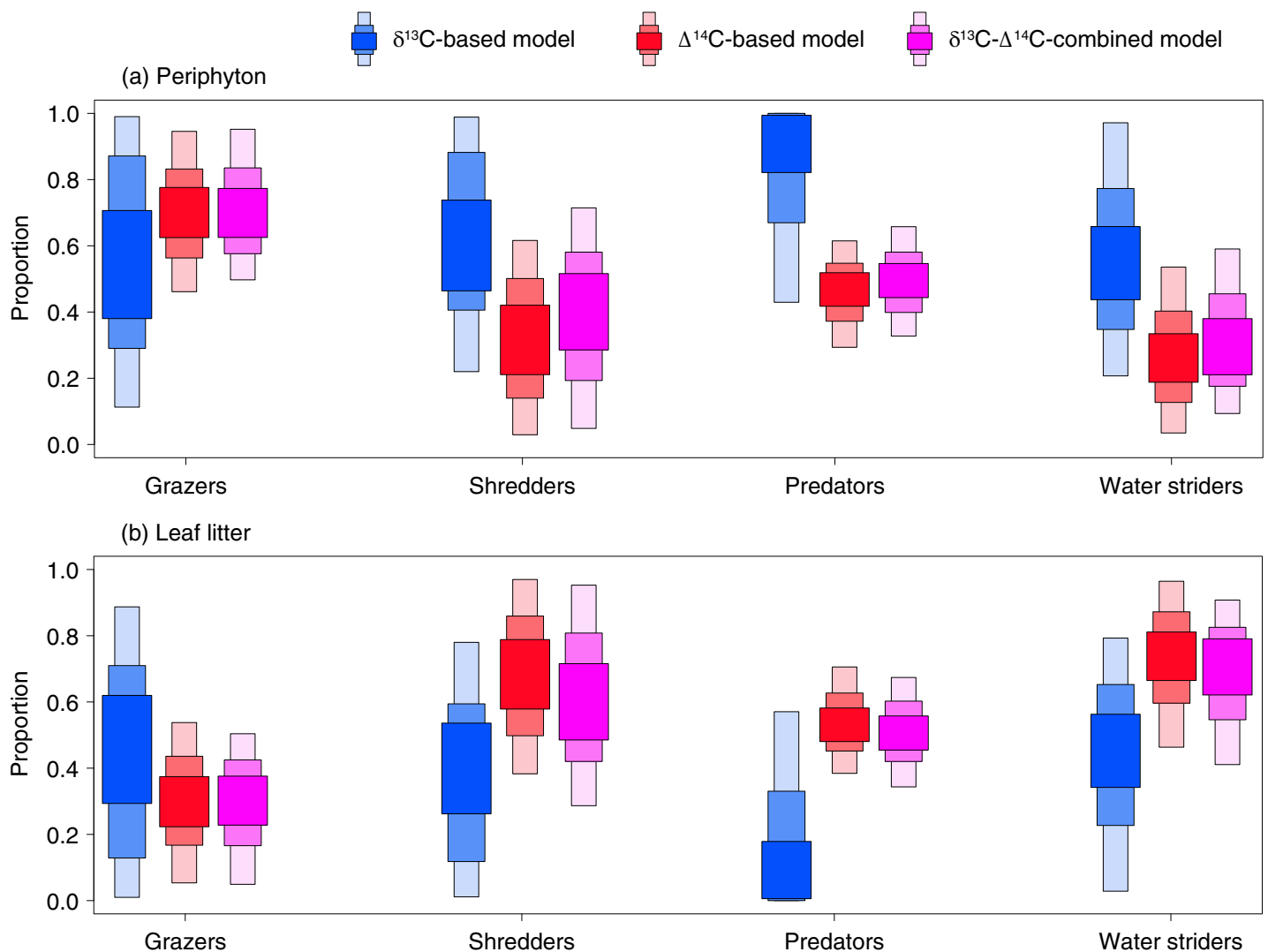


Fig. 3. Output of the SIAR mixing model (i.e., posterior probability distributions of proportion of invertebrate carbon derived from (a) periphyton and (b) leaf litter) using $\delta^{13}\text{C}$ (blue), $\Delta^{14}\text{C}$ (red), and $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ (purple). 25%, 75%, and 95% credible intervals are shown. Data from all five study sites were combined for modeling.

estimated by the $\delta^{13}\text{C}$ -based mixing model, whereas almost equal contributions of periphyton (mode: 49%; mean: 46%) and leaf litter (mode: 51%; mean: 54%) to predators were estimated by the $\Delta^{14}\text{C}$ -based mixing model. By contrast, the $\Delta^{14}\text{C}$ -based mixing model showed a higher contribution of leaf litter (mode: 74%; mean: 71%) to water striders (i.e., predators inhabiting streams but mainly consuming terrestrial invertebrates; McLean 1990) than that of periphyton, whereas the $\delta^{13}\text{C}$ -based mixing model indicated almost equal contributions of periphyton (mode: 53%; mean: 57%) and leaf litter (mode: 47%; mean: 43%) with a large uncertainty. Collectively, the feeding mode of each invertebrate was more consistent with the estimate calculated from $\Delta^{14}\text{C}$ than that from $\delta^{13}\text{C}$. Furthermore, incorporation of $\Delta^{14}\text{C}$ into the SIAR mixing model substantially reduced credible intervals of the

source contribution estimate for all functional feeding groups (Fig. 3). This improvement was achieved primarily because $\Delta^{14}\text{C}$ values of most invertebrates were between those of periphyton and leaf litter, whereas $\delta^{13}\text{C}$ values of most invertebrates were higher than those of the two sources (Fig. 2), suggesting that the invertebrates partly eat a food source not well represented by our cobble sampling.

Discussion

$\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ variability

The $\delta^{13}\text{C}$ value is widely used in stream food web studies, though the sensitivity of $\delta^{13}\text{C}$ to environmental variation (light, CO_2 , and water current) makes it difficult to differentiate algal from terrestrial vascular plant sources (Finlay et al. 1999;

Ishikawa et al. 2013). The $\delta^{13}\text{C}$ values of periphyton are determined by the magnitude of $^{13}\text{C}/^{12}\text{C}$ fractionation during uptake of DIC and by $\delta^{13}\text{C}$ of DIC (Finlay 2004; Doi et al. 2007; Ishikawa et al. 2012a). McKinley, Fox, and Skunk Creeks were heavily shaded by riparian forest, lowering photosynthetic activity of primary producers such as periphytic algae (Finlay et al. 2011). This shading increases isotopic fractionation during uptake of DIC, which decreases $\delta^{13}\text{C}$ of periphyton and grazers. The low $\delta^{13}\text{C}$ values of periphyton were relatively close to those of leaf litter (difference $< 3\text{‰}$) in McKinley, Fox, and Skunk Creeks, impeding discrimination of their relative contributions to invertebrate production (Figs. 2a–c). In previous studies, $\delta^{13}\text{C}$ values of DIC in McKinley, Fox, and Skunk Creeks were lower than those in Elder Creek and SF Eel River (Finlay 2003, 2004). Furthermore, water velocity is variable in these sites, creating a useful fine scale variation in $\delta^{13}\text{C}$ that allows discrimination of carbon sourced from riffles vs. carbon sourced from pools (Finlay et al. 2002). However, that variation obscures larger-scale (e.g., longitudinal) changes in the relative importance of algal vs. terrestrial carbon sources to stream consumers (Finlay et al. 1999; Finlay 2001).

The $\Delta^{14}\text{C}$ value is able to overcome the variation arising from environmental conditions when $\delta^{13}\text{C}$ is employed as a carbon tracer in stream ecosystems. In theory, the $\Delta^{14}\text{C}$ value is unaffected by isotopic fractionation of carbon associated with any physicochemical and biochemical process, because $\Delta^{14}\text{C}$ is internally corrected by $\delta^{13}\text{C}$ (Stuiver and Polach 1977). Our previous studies empirically showed that periphyton $\Delta^{14}\text{C}$ is independent of reach scale spatial heterogeneity within stream, such as open vs. shaded or riffle vs. pool environments, which causes variable fractionation against ^{13}C between DIC and periphyton (Ishikawa et al. 2012b). Dissolution of relatively ^{14}C -enriched atmospheric CO_2 into stream water resulted in the increase of periphyton $\Delta^{14}\text{C}$ as stream size increases (Fig. 1) (Ishikawa et al. 2014), except for the largest site SF Eel River, which might have higher erosion and availability of ancient organic carbon (e.g., kerogen) than the other sites we examined.

$\Delta^{14}\text{C}$ as a carbon tracer

Both the aquatic moss *F. antipyretica* and filamentous green algae *Cladophora glomerata* in the Eel River showed $\Delta^{14}\text{C}$ values intermediate between those of periphyton and leaf litter (Fig. 2), suggesting that they were affected by terrestrially derived particles either from (1) trapping within extracellular matrix (e.g., sticky muco-polysaccharide) on the surface of algal cell and/or (2) tangled in filaments or fronds. Therefore, we did not use these as aquatic end-members in the present study. It is possible that periphyton also contained terrestrial organic matter, although its contribution was at most 10% at a similar-sized stream (Ishikawa et al. 2015b). The $\Delta^{14}\text{C}$ values of algal biomarkers such as chlorophyll *a* in periphyton matrix, rather than the bulk $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$, may provide a

more accurate signature of the aquatic end-member (Ishikawa et al. 2015b).

Discrimination against ^{14}C during trophic transfer is also canceled in $\Delta^{14}\text{C}$ by the $\delta^{13}\text{C}$ correction, an advantage over the $\delta^{13}\text{C}$ method. We assumed the trophic discrimination factor for $\delta^{13}\text{C}$ as 0‰ because trophic levels of invertebrates were unknown to us in the present study. However, it might reach up to $\sim 1\text{‰}$ for consumers more than two trophic levels above basal resources. This uncertainty results in $> 30\%$ error of the source contribution estimate in the mixing model (Vander Zanden and Rasmussen 2001), given a relatively small $\delta^{13}\text{C}$ separation ($\sim 2.5\text{‰}$) between periphyton and leaf litter in the Eel River. Furthermore, the $\Delta^{14}\text{C}$ results provided detailed insights on the feeding ecology of stream invertebrates. First, remarkably low $\Delta^{14}\text{C}$ value of *Lepidostoma* spp. (-40.4‰) was found in the highly productive SF Eel River. This confirms substantial algal contributions to *Lepidostoma* spp. in sunny mainstem channels (in contrast to their diets in tributaries; J. M. Hood unpubl. data) (Fig. 2e; Finlay et al. 2002). The results reveal that the feeding strategy of assumed leaf-shredding consumers is flexible (Mihuc and Minshall 1995) and depends on in situ algal productivity or nutritional requirement at their growth stage. Second, the stonefly *Calineuria californica* $\Delta^{14}\text{C}$ in Fox Creek showed a large intraspecies variation ($> 70\text{‰}$) (Fig. 2c), suggesting that their diets may depend on their age or stage (cf. *Calineuria californica* is a semivoltine species; Siegfried and Knight 1978); on food availability; or on microhabitat. This pattern was not detected by $\delta^{13}\text{C}$, which showed a small intraspecies variation in *Calineuria californica* ($< 3\text{‰}$) and a small isotopic difference (2‰) between periphyton and leaf litter (Fig. 2c).

Radiocarbon also provides information of the age of organic carbon. In our study, the apparent radiocarbon age of invertebrates varied between modern and 479 yr B.P., depending on their food preference. Previous studies reported significantly old radiocarbon age (~ 5000 yr old) in particulate organic matter derived from soils in the Eel River watershed (Blair et al. 2003; Goñi et al. 2013). These observations suggest that approximately 10% of the soil organic matter contributes to carbon in stream invertebrates in the Eel River. However, when the old (^{14}C -depleted) organic matter is mineralized to inorganic carbon, fixed by algae, and eaten by invertebrates, this fresh but ^{14}C -depleted carbon is no longer old. This indirect transfer pathway must be separated from the direct feeding of old organic matter by invertebrates (Guillemette et al. 2017), which was not well characterized in the present study.

Although several studies (e.g., Caraco et al. 2010; Bellamy et al. 2017, 2019) have used $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ (and hydrogen isotopes) in their source mixing model, our results are the first direct comparison of the model performance with or without $\delta^{13}\text{C}$ or $\Delta^{14}\text{C}$. Since the Eel River watershed does not contain carbonate bedrock (McLaughlin et al. 2000) and has few deposits of carbonate minerals (Hahm et al. 2019), we expect

that streams in watersheds of limestone bedrock or other ^{14}C -depleted reservoirs (e.g., glaciers or permafrost) (Fellman et al. 2015; O'Donnell et al. 2020) will offer even greater improvement on source separation using $\Delta^{14}\text{C}$. It is hypothesized that the $\Delta^{14}\text{C}$ value of DIC is negatively correlated with the DIC concentration (i.e., [DIC]), assuming that the ^{14}C -depleted reservoirs are a major source of DIC. However, [DIC] strongly depends on stream discharge, and fluctuates greatly in small streams (Finlay 2003). Furthermore, other carbon sources such as atmospheric CO_2 dissolution and organic matter degradation provide ^{14}C -enriched carbon to DIC (Ishikawa et al. 2015a). Therefore, the above hypothesis should be tested in a variety of streams in the near future to evaluate the utility of [DIC] as a proxy for $\Delta^{14}\text{C}$ of DIC. The addition of other isotopes such as hydrogen and nitrogen to the mixing model may also improve the performance (i.e., lowering uncertainty) as well as resolution (i.e., adding another source such as the soil organic matter to the model, although its contribution to stream fauna is likely to be minor; Bellamy et al. 2017, 2019). However, since the ecosystem scale cycling of carbon and other elements (e.g., nitrogen) is sometimes decoupled in the metabolism of animals (Wolf et al. 2009), the isotopic routing may increase the uncertainty of the mixing model (Ishikawa 2018). In such a case, dual carbon isotopes would be the most straightforward tracer of carbon in ecosystems.

Limitations and perspectives

Our mixing model combining all the sites in the Eel River may have masked productivity-dependent diet shift as shown in a shredder *Lepidostoma* spp. Our approach was mainly intended to compare the performance of $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ as a tracer of aquatic vs. terrestrial contributions to stream food webs, but was not designed to quantify differences among sites. As a consequence, the specific estimates of sources presented in our mixing model results cannot be used to infer diet sources for any individual site. However, our approach enabled integration of not only the "intersite" $\delta^{13}\text{C}$ variation (productivity-dependent) but also "intrasite" $\delta^{13}\text{C}$ variation (microhabitat-dependent), the latter of which was not characterized in the present study due to the lack of replication for source data. The $\Delta^{14}\text{C}$ analysis is typically 10 times more expensive but requires less replication compared to the $\delta^{13}\text{C}$ analysis, because $\Delta^{14}\text{C}$ is less variable among microhabitats at reach scale (Ishikawa et al. 2012b).

Overall, we conclude that a complementary use of dual carbon isotopes is useful for separating aquatic and terrestrial resources. The $\delta^{13}\text{C}$ value is sensitive to fractionation that varies across riffles vs. pools or open vs. shaded environments for small invertebrates (Finlay et al. 1999, 2002). In contrast, the $\Delta^{14}\text{C}$ value, by correcting for $\delta^{13}\text{C}$ fractionation, offers a more robust tracer of carbon source at the reach scale (Ishikawa et al. 2012b). This method will be useful for studying large invertebrates and fish that migrate among microhabitats. Future studies should explore the variability in and controlling

factors on $\Delta^{14}\text{C}$ values in DIC and primary producers across space and time, which are now more poorly known than patterns and sources of variation in $\delta^{13}\text{C}$ values in primary producers. A global meta-analysis indicates that the $\Delta^{14}\text{C}$ values of DIC in world rivers ($-32 \pm 145\text{‰}$, mean and SD, $N = 209$; Marwick et al. 2015) are highly variable but are not always equilibrated with modern atmospheric CO_2 . We suggest that either (1) the presence of marine shales and mudstones in bedrock as shown in this study; (2) preferential respiration of old organic matter by heterotrophic bacteria (Hood et al. 2009; McCallister and del Giorgio 2012); or (3) significant contribution of groundwater to stream water (Fellman et al. 2014) is key for $\Delta^{14}\text{C}$ to separate stream water DIC from atmospheric CO_2 .

Finally, it should be noted that a combined use of $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ in the source mixing model may in some cases be a problem because the $\delta^{13}\text{C}$ value is normalized to -25‰ in the $\Delta^{14}\text{C}$ value (Eq. 2). To be exact, the original definition of $\Delta^{14}\text{C}$ for unknown samples does not assume multiple carbon sources (Stuiver and Polach 1977). If two sources are assigned, $\delta^{13}\text{C}$ is represented as a linear equation of $\delta^{14}\text{C}$, and thus $\Delta^{14}\text{C}$ is represented as a quadratic equation of $\delta^{13}\text{C}$ or $\delta^{14}\text{C}$ (Eq. 2). Therefore, the combined mixing model based on a linear relationship between $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ may not apply when the $\delta^{13}\text{C}$ value of one end-member is far different from that of the other (Ishikawa et al. 2013). However, the quadratic term is usually negligible due to a very small coefficient, which allows us to approximate $\Delta^{14}\text{C}$ as a linear function of $\delta^{13}\text{C}$ (Ishikawa et al. 2013). In our results, the performance was similar between the $\Delta^{14}\text{C}$ -based model and the $\delta^{13}\text{C}$ - $\Delta^{14}\text{C}$ -combined model (Fig. 3). We recommend that future researchers carefully check to see whether the $\delta^{13}\text{C}$ internal correction in $\Delta^{14}\text{C}$ affects the final model output.

Ethical statement

All organisms used in the present study did not include any endangered species, and were exclusively dedicated to the isotope analysis.

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Conflict of Interest

None declared.

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