Title: Cryptic chemoautotrophic and methanotrophic processes complicate the use of carbon stable isotopes to understand freshwater productivity

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Significance Statement

The use of carbon stable isotopes has contributed to many important discoveries regarding the base of consumer production in freshwater ecosystems. There is increasing recognition for the prevalence of anoxic conditions and the contributions of methane-derived carbon to freshwater food webs, highlighting the potential for methanotrophy and chemoautotrophy to complicate interpretations of $\delta^{13}C$ values in consumer biomass. These reduced substrates are sometimes cryptic, or present at such low concentrations that they might not provide an obvious carbon source, especially in rivers which are typically conceptualized as well-oxygenated. At most, chemoautotrophy and methanotrophy might be important, yet overlooked, carbon sources and at least, even a small amount of these isotopically lighter contributions could complicated assessments of basal carbon sources to river food webs. An improved application of carbon-based stable isotope mixing models to freshwater ecosystems, and subsequent inferences of dietary resources, is limited by three major assumptions reviewed herein. These assumptions include: representation of dietary end members using bulk sampling of organic matter sources, estimation of fractionation rates associated with microbial oxidation reactions, and the decision of whether or not to include proxies for the heavily fractionated biomass of chemoautotrophs and methanotrophs. With improved recognition and continued study of these assumptions, the conceptualization of basal carbon sources can be expanded to include the redox complexity of both lentic and lotic systems, and could expand how we conceptualize the boundaries of river food webs. This has implications for recognition and management of habitat heterogeneity in freshwaters.

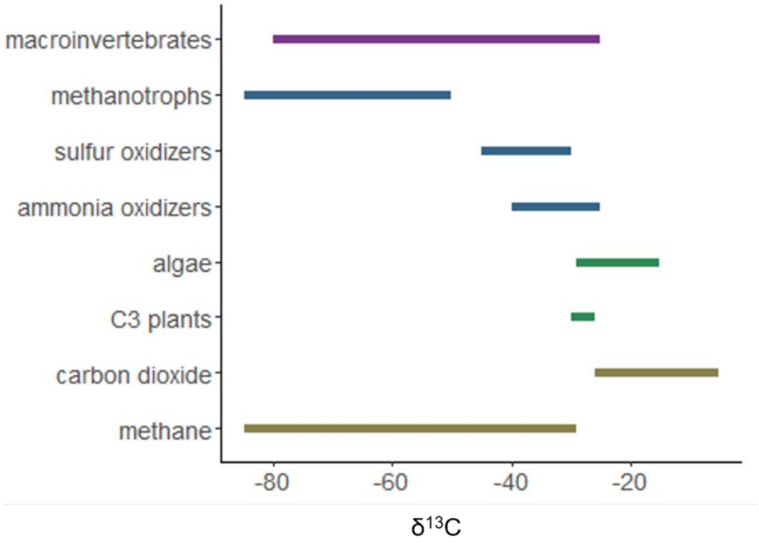

Figure 1. Macroinvertebrates, or secondary consumers, represent a mixture of available food sources. In systems where macroinvertebrate $\delta^{13}C$ values are more depleted than bulk organic matter sources, chemoautotrophic or methanotrophic contributions to the food web are clear, and thus generally represented by proxy in understanding isotopic values. However, chemoautotrophy and methanotrophy might still contribute to the food web even when consumers are not notably depleted, thereby potentially acting as cryptic carbon sources that complicate understanding of $\delta^{13}C$ values. Range values are supported by Kohzu et al. 2004, Jones and Grey 2011, DelVecchia et al. 2016, Grey 2016, and DelVecchia et al. in review.

Carbon isotopes are commonly used to determine the energetic basis of consumer production in freshwater systems. In lotic systems, carbon stable isotope ratios ($^{13}C/^{12}C$ or $\delta^{13}C$) have commonly been used to distinguish contributions of locally-produced, photosynthetically fixed carbon from external contributions originating terrestrially (e.g. Finlay 2001, Allen et al. 2024). The distinction between autochthonous and allochthonous $\delta^{13}C$ values has been used to refine conceptualizations of energy flow and food web structure in streams and rivers (Pingram et al. 2012). However, it is possible that interpretation of food web $\delta^{13}C$ values has been limited by a prevailing assumption that streams and rivers are consistently well-oxygenated, and thus that contributions of alternative processes of carbon fixation (methanotrophy and chemoautotrophy) are limited.

Although streams and rivers have typically been conceptualized as well-oxygenated systems fueled by photosynthetically-fixed carbon, there is increasing recognition of anoxia (Blaszczak et al. 2023), methane cycling (Stanley et al. 2016, Rocher-Ros 2023), and methane-derived carbon contributions to food webs (Grey 2016). Indeed, methane contributions to food webs have been evidenced by anomalously low $\delta^{13}C$ values of consumer biomass (Jones and Grey 2011, Grey 2016). Methane in freshwater is generally produced in anoxia and oxidized rapidly by methanotrophs where oxygen is present. Similarly, ammonia and hydrogen sulfide can accumulate in anoxic conditions and be oxidized at a redox interface. All of these processes fractionate the carbon pool and result in isotopically-differentiated organic carbon contributions to the food web, but often occur without measurable concentrations of the reduced substrates involved or without clear inclusion of these resources in bulk organic matter. I argue in this essay that chemoautotrophic and methanotrophic processes could still contribute to productivity, and thus act as cryptic carbon sources that complicate the inferences that can be drawn from $\delta^{13}C$-based mixing models or inferential frameworks. Methods development addressing the assumptions listed herein might improve our understanding of the heterogeneity of energy sources in freshwaters.

Stable isotope approaches are best applied with an underlying comprehension of the system's ecology, because it is crucial that mixing models consider empirical or estimated values for all potential sources (end members) to a given mixture in order to obtain representative results (Parnell et al. 2010, *Stable Isotope Ecology* n.d.). These applications are thus constantly subject to a series of assumptions governing their application, and the accuracy of source contribution estimates is limited by those assumptions. Herein I identify three major assumptions that limit application and interpretation of $\delta^{13}C$ values in freshwater ecosystems given an increased recognition for methane-derived carbon contributions. The goal of recognizing these assumptions is to identify avenues for research that could refine application of carbon-based mixing models, and to advocate consideration of a broader suite of possibilities that could explain freshwater $\delta^{13}C$ values. The assumptions are as follows:

1. That invertebrate food resources are represented by bulk sampling of organic matter
2. That fractionation rates from highly-cited empirical studies are applicable to all systems
3. That the potential for a given redox contribution is related to the concentration of corresponding substrates

Assumption 1: That invertebrate food resources are represented by bulk sampling of organic matter

Stable isotope (SI) analyses are commonly used to distinguish between carbon sources to freshwater food webs, most often to distinguish between autochthonous and allochthonous resources. However, studies of consumer biomass have revealed that bulk organic matter sampling cannot consistently reveal all food sources to consumers. Recently, Allen et al. (2024) in their meta-analysis of global allochthony, found that 2138/2730 total observations of allochthonous diet contributions to stream and riparian consumers were estimated via SI mixing models, with the remaining estimates coming from gut content analyses. In the cited studies, samples of potential sources were generally collected from stream periphyton and other bulk organic matter (OM) classes, as well as from the surrounding terrestrial environment. The assumption that OM sampling can represent all possible carbon contributions to consumers has traditionally seemed valid considering that the $\delta^{13}C$ values for consumers are often in a range that could be explained by photosynthetically-fixed carbon at the base of the food web, whether it be allochthonous or autochthonous. Consumer biomass with δ^{13}C values more negative (depleted) than any sampled organic matter sources suggest contributions of methane-derived carbon, or carbon fixed via other chemoautotrophic processes. Other chemoautotrophic pathways include ammonia or sulfur oxidation which also assimilate CO_2 through the Calvin cycle (Sakata et al. 2008). Methanogenesis, methanotrophy, ammonia oxidation, and sulfur oxidation all result in microbial biomass depleted by 15 to 60‰ relative to ambient CO_2 pools (Figure 1).

Many studies which have documented methane-derived carbon or chemosynthetic contributions to consumer biomass, particularly through δ^{13}C values, did not have correspondingly depleted bulk OM samples. The depleted δ^{13}C values of consumer biomass suggested a need to model proxies for alternative carbon sources. For example, in the Nyack Floodplain of Montana, USA, stoneflies collected from the alluvial aquifer had δ^{13}C values as low as -79 ‰, conclusively showing a methane-derived carbon contribution, yet OM ranged to only -39 %, over multiple years of sampling (DelVecchia et al. 2016). The same phenomenon occurred in a stream in Hokkaido, Japan, with invertebrate δ^{13}C values ranging to -55.3 ‰ and OM signatures of -28 ‰, as well as in chalk streams in England where caddisflies had lower δ^{13}C values (-45 ‰) than bulk epilithon ranging to -40 ‰ (Kohzu et al. 2004, Sampson et al. 2019). In both of these other cases with less-depleted invertebrate signatures, the authors also deduced a methane-derived carbon contribution.

Chemoautotrophic contributions have similarly been concluded: in the Cement Creek Floodplain in Colorado, USA, consumers had δ^{13}C values in ‘the contentious zone’, or in the -40 to -30 ‰ range which could be explained by a suite of possibilities (Grey 2016), and periphyton and OM signatures ranged to -29.8 ‰ (DelVecchia et al. In review). Estimates of methanotrophic or chemoautotrophic carbon contributions to consumer biomass were derived from mixing models that were parameterized by representing the δ^{13}C and Δ^{14}C of methane oxidizing bacteria, sulfur oxidizing bacteria, and ammonia oxidizing bacteria end-members using literature-based fractionation rates and the δ^{13}C and/or Δ^{14}C values of the ambient dissolved CO_2 and CH_4. The authors concluded that invertebrates were obtaining carbon fixed through ammonia or sulfur oxidation. In all of these cases and others (e.g. Hershey et al. 2006, Jones et al. 2008, Deines et al. 2009), the authors concluded that invertebrates must be obtaining food resources not evidenced by bulk organic matter samples. In some studies, findings leveraged the $\delta^{13}C$ signatures as well as $\delta^{34}S$ signatures (Doi et al. 2006, Deines et al. 2009, Karube et al. 2012), $\delta^{2}H$ signatures (Doi et al. 2006, Karube et al. 2012), phospholipid fatty acid analysis (Kiyashko et al. 2001), or gut content analysis to parse out diet contributions rather than using $\delta^{13}C$ values alone.

Importantly, the observation that consumer biomass had more negative $\delta^{13}C$ values than organic matter served as a cue to test the possibility of either a methanotrophic or chemoautotrophic carbon contribution to the food web. However, it is distinctly possible that these alternative modes of carbon fixation contribute to food webs (even if to a limited degree) even when consumer biomass is not more depleted in $\delta^{13}C$ values than sampled organic matter. The same phenomenon could be occurring within the bulk organic matter pool, yet it still remains a mystery why consumers have biomass values more reduced than bulk OM. This has implications for general interpretation of $\delta^{13}C$ values in consumer biomass, including estimates of allochthony. Even a minimal contribution of methanotrophic carbon (e.g. 1%) within the range shown in Figure 1 could alter estimates of allochthony drastically, depending on the differentiation between terrestrial and aquatic OM values.

<u>Assumption 2</u>: That fractionation rates from highly-cited empirical studies are applicable to all systems

In those cases where consumer $\delta^{13}C$ values were more depleted than any sampled food sources, other microbial end members were represented via proxy using literature fractionation rates and measurements of either $\delta^{13}C$-CH_4 or $\delta^{13}C$-CO_2. These literature fractionation rates enable the estimation of $\delta^{13}C$ for a given functional taxa based on the extent that taxa might preferentially assimilate lighter carbon. However, published fractionation rates, despite their implications for food web modeling, are sparse. The lack of repeated experiments both within and across taxa, as well as of clear growth stage to fractionation relationships limits accurate representation of microbial end members in mixing models. For example, multiple studies assessing a contribution of methane-derived carbon to consumers cite the work of Sakata et al. (2008) to account for an ammonia-oxidizing end member (Trimmer et al. 2009, 2010, Appling 2012, DelVecchia et al. 2016, Sampson et al. 2019, Pacioglu et al. 2022). Sakata et al. report δ^{13}C fractionation rates of approximately -20 ‰ in average biomass of *Nitrosomas europea*. Information on the δ^{13}C fractionation rate of other taxa is lacking. Similarly, multiple studies represent or cite the δ^{13}C value of a sulfur-oxidizing end member using the fractionation rate published by Ruby et al. (1987) (Sarbu et al. 1996, Kiyashko et al. 2001, Wadham et al. 2004, Trimmer et al. 2009, DelVecchia et al. 2016, Sampson et al. 2019). Ruby et al. showed that biomass δ^{13}C values were depleted by -24.6 to 25.1 ‰ relative to ambient DIC using a *Thiomicrospira spp.* strain isolated from a hydrothermal vent and a *Thiobacillus neapolitanus* isolated from marsh soils. The fractionation rate for methane oxidation in many of these same studies is often cited from Summons et al. (1994) and more recently, Templeton et al. (2006). Summons et al. cultured *Methylococcus capsulatus* and *Methylomonas methanica*, observing fractionation rates in relation to growth phase. This approach to fractionation revealed an additional complication in estimating δ^{13}C values of methanotroph biomass – fractionation from methane substrate varied from -16 to -30 ‰ depending on whether methanotrophs were in a stationary or exponential growth phase. This information is seemingly lacking for other taxa and it is possible that δ^{13}C estimates of end members are subject to far higher variability than is modeled using static fractionation factors (e.g. the MixSIAR framework; Stock et al. 2018).

The empirical measurement of microbial fractionation rates associated with particular processes is intensive and difficult, requiring controlled microcosms in which a given microbial taxa is isolated and cultured, and the change in δ^{13}C between carbon resources (e.g. the CO_2 or CH_4 pool) and microbial biomass is carefully measured over time. This difficulty explains why few papers have measured fractionation rates of particular processes. However, these experiments could prove extremely useful in the determination of proxies that are included in mixing models or inferences based on δ^{13}C values.

Assumption 3: That the potential for a given redox contribution is related to the concentration of corresponding substrates

The decision of which end-members to include in mixing models is subjective, yet determines the integrity of model results (Parnell et al. 2010, *Stable Isotope Ecology* n.d.). This is a particular problem in stream systems because they have typically been viewed as well-oxygenated, and thus the potential for redox interfaces in which these fractionating microbial processes is often overlooked. These redox interfaces are places where methanotrophy and chemoautotrophy might be likely to occur due to the presence of both oxygen and reduced substrates (e.g. sulfide, ammonia, nitrite, methane) (Sakata et al. 2008). The presence of anoxic habitats and timespans, whether in the surface channel or in the hyporheic zone, is increasingly recognized in lotic systems through direct observation of dissolved oxygen concentrations and the presence of biogenic methane (Stanley et al. 2016, Malison et al. 2020, Blaszczak et al. 2023, DelVecchia et al. 2023). Although this recognition of anoxia in rivers is growing, the reduced substrates associated with chemotrophy and methanotophy are rarely present in high concentrations, and these concentrations are not necessarily suggestive of inclusion in mixing models (e.g. DelVecchia et al. 2016, Sampson et al. 2019). Reduced substrates could be oxidized rapidly and still contribute to microbial biomass and thus food resources available to consumers, making these oxidation processes cryptic in study of the aquatic food web (e.g. Shelley et al. 2014).

As a stark example, DelVecchia et al. (2016) found clear evidence of methane contributions to hyporheic consumers even when methane concentrations were consistently below detection limit. These low methane concentrations could have been an artifact of sampling method, as isolation of substrates present in anoxia can be intensive. Or, methane concentrations might have truly been low because methane was rapidly oxidized. In other words, ambient concentrations provide only a snapshot in time rather than an indication of an ongoing process, whereas consumer biomass integrates food resources over the time period of tissue turnover. It is possible and likely that despite low substrate concentrations, methanotrophy and chemoautotrophy serve as cryptic carbon sources, silently influencing mixing model results even when it is assumed that these processes are not occurring. By measuring rates of relevant redox processes rather than just concentrations, it might be possible to understand how these processes might contribute to productivity. An excellent example of how a suite of biogeochemical studies can support inferences of food web contributions is provided by the work of Sampson et al. 2019, in which their inferences based on δ^{13}C values were supported by past work measuring rates of redox processes. Even when process measurements are not possible, the use of additional isotopes (e.g. δ^{2}H and δ^{34}S) to distinguish other potential redox contributions tends to be more affordable, yet helps to parse the uncertainty inherent in the interpretation of consumer δ^{13}C values.

Conclusion

Rivers and streams are not exclusively the well-mixed, well-oxygenated, lotic systems that are seen from the surface channel. As recognition for the physical and biogeochemical heterogeneity of rivers grows, so does the need for better ways to quantify the importance of ecological processes that might occur at the redox interface. Both chemoautotrophic and methanotrophic contributions are possible in many systems, regardless of concentrations of related substrates, δ^{13}C values of consumers, or assumptions of oxygenation. These contributions are important to recognize and quantify because in some cases, they might provide a significant source of carbon to consumers. In other cases, even minimal contributions of these resources, if unaccounted for, could affect estimates of allochthony or the food web energy base. Current applications of carbon-based mixing models to understand food webs could be improved by a recognition for potential cryptic carbon contributions, whether or not redox heterogeneity is obvious.

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