Temporal Analysis of River Food Webs

By

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Submitted to the graduate degree program in Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Temporal Analysis of River Food Webs

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Abstract

Rivers and their tributaries are the arteries of the planet, pumping freshwater to wetlands and lakes and out to sea. Understanding energy flow up trophic levels, nutrient cycling pathways, and relative importance of terrestrial and aquatic carbon sources supporting aquatic consumers in large river food webs is essential in planning for wildlife conservation, environmental protection, and floodplain management. The principal goal of my dissertation is to understand better the factors controlling the complexity of river food webs through time.

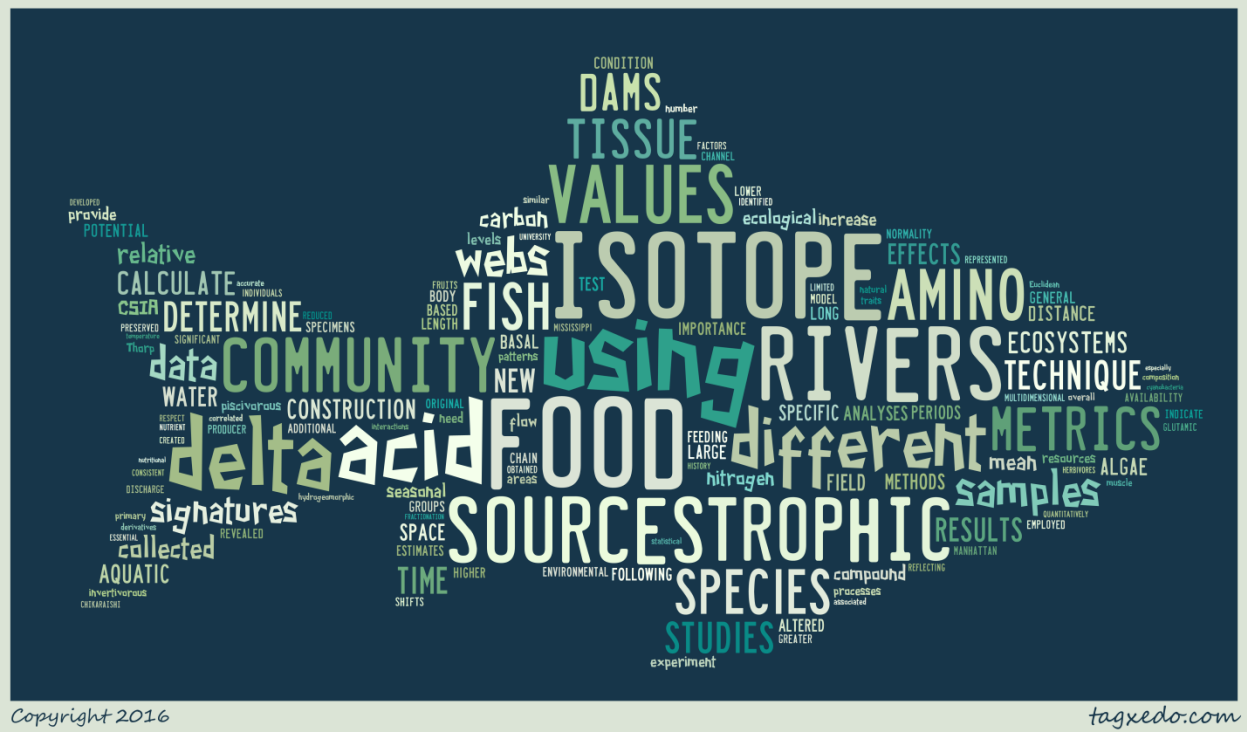
At a shorter time scale, I first look at how season and food availability affect fish in rivers. I employ bulk tissue stable isotope analysis to determine trophic position of fish in the field, over different seasons, and fish in the lab, under different amounts of nutrient stress.

Scientists continue to debate which factors control the relative importance of organic sources fueling food webs of large rivers. Resolution of this debate requires a new technique: identifying food sources and trophic position using traditional bulk-tissue stable isotope techniques is difficult because of spatiotemporal variability of carbon sources, mixing model problems with too few tracers, and unavailability of reliable basal signatures.

In the remaining chapters of the dissertation, I utilize a new technique, applying nitrogen and carbon stable isotope analysis of amino acids to samples to determine trophic position and carbon food sources over time. First, in Chapter 2, I demonstrate the utility these new methods in a controlled feeding experiment in the laboratory, determining fish trophic positions. I show that the new methods seem to offer more accuracy and precision in trophic position estimates when compared to more traditional methods of bulk tissue isotope analysis. With these new analytical methods, I propose multidimensional metrics for use with compound specific analyses of food webs, as well as other multidimensional community measures (e.g., fatty acids, ordinal

traits) in Chapter 3. Then, I evaluate long-term historical changes in trophic position (chapter 4) and food sources (chapter 5) of fish museum specimens using amino acid stable isotope analyses of both the Mississippi and Ohio rivers.

Figure 0-1. Word cloud of Bowes Dissertation: Temporal Analysis of River Food Webs, made with http://www.tagxedo.com, and used here under a Creative Commons Attribution-Noncommercial-ShareAlike License 3.0.



Acknowledgements

This dissertation would not have been possible without the help and support of many people and sources of funding.

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Of course, nothing in science would be possible without funding, and I am grateful to the following agencies for providing me with the opportunity to pursue my research ideas. The University of Kansas Department of Ecology and Evolutionary Biology, Kansas Biological Survey, Association for Women Geoscientists Osage Chapter, University of Kansas Center for Undergraduate Research, NSF EAGER grant #1249370 to James Thorp, NSF DDIG grant #1502017 to Rachel Bowes and James Thorp, NSF Macrosystems Biology grant #1442595 to James Thorp. Work was also supported by the US Environmental Protection Agency under Star Grant #RD-83244201 to James Thorp and Michael Delong. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funders.

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Introduction

Rivers and their tributaries are the arteries of the planet, pumping freshwater to wetlands and lakes and out to sea. They flush nutrients through aquatic ecosystems, keeping thousands of species alive, and sustain fisheries worth billions of dollars. Rivers are also the lifeblood of human civilizations, supplying water to cities, farms, and factories. Rivers provide shipping routes around the globe, and provide food, recreation, and energy to human societies. Hydroelectric plants built from bank to bank harness the power of water and convert it to electricity. The importance of large rivers to societies is easily recognizable because many modern and ancient population centers are located along large rivers, tributaries, streams, and deltas. However, rivers are also often the endpoint for much of our industrial and urban pollution and runoff. The result: polluted drinking water sources, the decline of aquatic species, and coastal dead zones caused by fertilizer and sewage overload.

Documenting changes in aquatic ecosystems is notoriously difficult; however, food webs are a central organizing theme in ecology. Food webs are special descriptions of biological communities focused on trophic interactions between consumers and resources. They provide a means of analyzing interrelationships among community structure, stability, and ecosystem processes, and how these attributes are influenced by environmental change and disturbance. Well-functioning food webs are fundamental in sustaining rivers as ecosystems and maintaining associated aquatic and terrestrial communities.

The principal goal of my dissertation is to understand better the factors controlling the complexity of river food webs through history – a goal which requires development and testing of a new analytical method.

Stable isotopic analysis of biochemically important elements is a widely used tool in the study of ecology. Isotopic fractionation patterns in carbon and nitrogen are used to determine trophic position, nutrient pathways, and sources of primary production in numerous contexts. Traditional techniques rely on measurements made of bulk samples of tissue. The first chapter of the dissertation assesses one significant issue ecologists face when using bulk tissue stable isotope analysis. Isotopic ratios of nitrogen are often used in food web studies to determine trophic position, with greater ratios of $^{15}N/^{14}N$ ($\delta^{15}N$) usually considered indicative of higher trophic position. However, fasting and starving animals may also show a progressive increase in $\delta^{15}N$ over time as they catabolize their own tissues. To determine the importance of starvation, I conducted a laboratory experiment in conjunction with a seasonal field experiment on the Kansas River. Overall body condition resulting from reduced food consumption explained 44% and 53% of the variability in ^{15}N for field and lab fish, respectively.

Recently developed methods using isotopic signatures of individual chemical compounds provide a means of achieving deeper understanding in a variety of inquiries. Amino acids, as the building blocks of proteins, are the dominant nitrogen-bearing biomolecules and are a major constituent of all life. Patterns of isotopic fractionation during synthesis and transformations of these compounds record a variety of information about their environmental history. The second chapter of the dissertation demonstrates the utility and benefits of compound specific amino acid stable isotope analysis in measuring trophic position and food chain length. I conducted a cost-benefit analysis of both isotopic (bulk and amino acid) techniques in a controlled laboratory feeding experiments. My experiment demonstrated that the amino acid technique more accurately identified the true trophic position and food chain length, with significantly less variability around mean values for each consumer. Trophic position determinations derived from

measurements of amino acid $\delta^{15}N$ benefit from that fact that a subset of these compounds directly record ecosystem isotopic baseline values. Moreover, the amino acid technique solves many of the difficulties traditionally facing ecologists using bulk isotope analyses (i.e. the spatiotemporal variability of carbon sources and unavailability of reliable basal signatures).

The third chapter of my dissertation proposes a multidimensional metric for use with compound specific analyses of food webs, as well as other multidimensional community measures (e.g., fatty acids, ordinal traits). Using Manhattan distance, I create n-dimensional plots and metrics in which to quantitatively characterize community-wide aspects of trophic structure, niche space, and food sources. I demonstrate the utility of these newly-developed multidimensional metrics through analysis of amino acid compound-specific stable isotopes. The method provides increased resolution, and reveals new dimensions compared to traditional analytical frameworks.

Finally, the fourth and fifth chapters evaluate historical changes in trophic position (chapter 4) and food sources (chapter 5) of fish museum specimens using amino acid stable isotope analyses of both the Mississippi and Ohio rivers. I found significant opposite shifts in trophic positions and different variability in food sources (in particular algae) used by fish in the Ohio and Upper Mississippi rivers over a 50-yr period that were linked, by change-point analysis, with major alterations to habitat structure resulting from construction of low-head dams. These two rivers naturally vary in hydrogeomorphic complexity (anastomosing vs constricted), and their discharge patterns differ both from each other (seasonal vs yearly operation in some cases) and from those characterizing high dams. It is not surprising, therefore, that factors controlling trophic position and food sources apparently vary between different types of dams and river structures.

Chapter 1*

Less means more: isotope ratios in nutrient stressed fish

*Bowes, R.E., Lafferty, M.H., and J.H. Thorp. 2014. Less means more: nutrient stress leads to higher $\delta^{15}N$ ratios in fish. *Freshwater Biology*, 59: 1926-1931.

Abstract

Isotopic ratios of nitrogen are often used in food web studies to determine trophic position (including food chain length) and food sources, with greater ratios of $^{15}N/^{14}N$ ($\delta^{15}N$) usually considered indicative of higher trophic position. However, fasting and starving animals may also show a progressive increase in $\delta^{15}N$ over time as they catabolize their own tissues. To determine the importance of starvation, we conducted a four-month laboratory experiment testing effects of starvation on body condition and isotope ratios in the muscle tissue of freshwater guppies (*Poecilia reticulata*). We also compared laboratory results and conclusions with analyses of body condition and isotope ratios in various small species of fish collected in four seasons from the Kansas River in northeastern Kansas, USA. Fish starved in our lab experiment had significantly higher ^{15}N values and poorer body condition than those fed more regularly. The diverse group of fish species collected in summer (July) from the Kansas River had higher weight-to-length ratios and lower ^{15}N values than those retrieved in other seasons. Overall body condition resulting from reduced food consumption explained 44% and 53% of the variability in ^{15}N for field and lab fish, respectively. These results are applicable to a wide variety of food web research but are especially pertinent to studies of organisms that undergo large changes in life history, dormancy, extended fasts, or periods of significant nutritional allocation to young.

Introduction

An important metric of environmental health within ecosystems is food web structure because it reflects species richness, lifestyle diversity, and trophic interactions inherent among organisms. Recent studies have enabled us to investigate questions such as the following. What is the relative importance of ecosystem size, productivity, disturbance, and habitat complexity in controlling food chain length (e.g., Post & Takimoto 2007, Sabo et al. 2009, McHugh et al. 2010)? What factors control the relative importance to aquatic food webs of autochthonous vs. allochthonous carbon (e.g., Thorp et al. 1998, Doucett et al. 2007, Dudgeon et al. 2010, Winemiller et al. 2011)? And, what are the effects of anthropogenic impacts on food chain length and web complexity? Progress in answering these questions has greatly benefitted from use of stable isotope analysis (Peterson & Fry1987; Post, 2002; Roach et al. 2009; Winemiller et al. 2010, 2011).

Stable isotopes integrate food sources and reveal what an organism has generally assimilated over longer time scales compared to conclusions from shorter-term techniques, such as gut content and behavioural analyses. As organic matter is processed by cells, there is a tendency for selective retention of heavier isotopes and loss of lighter isotopes through chemical and physiological processes such as excretion or respiration. The change in relative abundance of heavy-to-light nitrogen isotopes ($\delta^{15}N = {}^{15}N/{}^{14}N$) can be used to estimate trophic position. The $\delta^{15}N$ of a consumer is typically considered to increase or become enriched by 3-4‰ relative to its diet (Deniro & Epstein 1981).

Interpreting food sources and trophic position based on $\delta^{15}N$ may be misleading, however, because tissues of fasting and starving animals may also show a progressive increase in $\delta^{15}N$ as body mass decreases (Hobson et al. 1993; Cherel et al. 2005). This occurs because

starving animals undergo catabolic processes, where they literally "live on their own meat" (Waterlow 1968). Indeed, a growing number of studies have proposed that the $\delta^{15}N$ ratio could be used as a general index of nutritional stress. This ^{15}N enrichment has been found in arctic ground squirrels (*Spermophilus parryii*) due to fasting (Ben-David et al. 1999), Japanese quail chicks (*Coturnix japonica*) raised on different feeding regimes (Hobson et al. 1993), hibernating American black bears (*Ursus americanus*) (Lohuis, Harlow & Beck 2007), and even humans suffering from anorexia nervosa (Mekota et al. 2006).

Food web studies using stable isotopes sometimes assume that seasonal effects or pronounced differences in food web structure are due to changes in relative choices of prey type (Cherel et al 2007). However, these fluctuations could also result from starvation. If stable isotope ratios are indeed significantly affected by starvation or food availability, how do we interpret these results and differentiate them from an actual modification in choice of prey, and exactly how much are isotope values shifted?

We analyzed the effects of nutritional stress (reduced food resources through starvation) on the nitrogen stable isotope composition of fish from the field (mixed riverine assemblage) and in a laboratory experiment with guppies (Poeciliidae, *Poecilia reticulata* Peters, 1859). We hypothesized that nutritionally stressed individuals would have significantly higher $\delta^{15}N$ ratios than more regularly fed fish (cf., Hobson et al. 1993; Cherel et al. 2005).

Methods

Laboratory Feeding Experiment

We examined effects of feeding schedule on $\delta^{15}N$ of muscle tissue in 30 laboratory raised guppies (*Poecilia reticulata*). Offspring from a laboratory breeding population of these fish were

fed *ad libitum* TetraFin Goldfish Flakes (42% crude protein, 8% crude fat, 2% crude fiber; http://www.tetra.net) for a period of four months on one of three schedules: daily, every three days, and every six days. Fish were adults (sexually mature, 25-40mm in length) and kept under a 12 h light/12h dark cycle at ~21°C. Tanks were filtered continuously and cleaned weekly, to ensure no algae or debris accumulated. No experimental fish perished during the study.

Field Study

We determined $\delta^{15}N$ of muscle tissue of field populations of small fish seined in 2013 from shoreline areas of the Kansas River near Lecompton, Kansas, USA (39.049664 N, 95.387214 W). Fish were sampled approximately every three months throughout the year (October 29, February 1, May 1, and July 29). During this period, we collected a total of 234 small fish for stable isotope analysis. These were a mixed assemblage of sand shiners (*Notropis stramineus*), red shiners (*Cyprinella lutrensis*), fathead minnows (*Pimephales promelas*), juvenile bluegill sunfish (*Lepomis macrochirus*), spotfin shiners (*Cyprinella spiloptera*), and mosquitofish (*Gambusia affinis*). The community composition varied somewhat among sample dates, but mosquitofish and spotfin shiners were typically most abundant, and thus the overwhelming majority used in the isotope analysis. All fish used in the analysis were identified to species, weighed, and total length measured to determine body condition in terms of weight-to-length ratios. Only fish of sufficient size were used in the isotope analysis (25–50 mm in length).

Stable Isotope Analysis and Trophic Position

At the end of the lab experiment and following each field sampling period, fish selected for isotopic determination were euthanized by ice water bath, body condition assessed, and then stored in 75% ethanol. Fish were identified to species and their body condition determined from weight (grams) measurements divided by the total length (millimeters) of the fish to give an overall ratio of body condition.

To determine isotope ratios, following the fish being stored for one week in ethanol, we removed a small plug of muscle from the body muscle tissue below the dorsal fin, rinsed it with deionized water, and placed it in pre-combusted glass vials. Tissue samples were dried in an oven at 60°C for 48 hr and then ground into a fine, homogenized powder using a Wig-L-Bug® Mixer/Amalgamator. Subsamples (2.0-3.0 mg) were packaged into 4 x 6 mm tin capsules and held in desiccators until submitted for bulk-tissue stable isotope analysis.

We evaluated the nitrogen isotopic composition of fish muscle tissue and powdered laboratory flake food using a ThermoFinnigan MAT 253 continuous flow system mass spectrometer (W. M. Keck Paleoenvironmental and Environmental Stable Isotope Laboratory, University of Kansas). The data for each sample included total N and $\delta^{15}N$ values. The $\delta^{15}N$ values were determined based on the relative difference in isotopic ratio between the samples and known standards, as represented by the following equation:

$$\delta X = ((Rsample / Rstandard) - 1) \times 1000$$

where X is ^{15}N, the corresponding ratio is $R = {}^{15}N/{}^{14}N$, and atmospheric nitrogen was used as the N standard. All isotope ratios are given in per mil (‰).

The $\delta^{15}N$ signature of the muscle tissue collected from a fish typically integrates what it has eaten over the past three months (Madigan et al. 2012; Maruyama et al. 2001; Xia et al. 2013). Therefore, the signature of fish collected in each of the different seasons in the field

portion of the study represents an integration of the resources consumed since the previous season of collection.

Statistical Analyses

We determined the effects of starvation and seasonality on body condition as well as nitrogen isotope ratios using ANOVA test (Minitab14 statistical software) with $\alpha = 0.05$. The relationship between nitrogen isotope ratios and body condition, associated with different seasons and feeding regimes, was determined by regression analysis and ANCOVA. The data were checked for normality and homogeneity of variances.

Results

The muscle tissues of guppies from our laboratory experiment fed every six days were significantly enriched in ^{15}N (Tukey's post-hoc test; ANOVA, $F_{2,19} = 16.98$, $p < 0.0001$; Fig. 1a). Fish fed every six days also had a significantly lower body condition than the fish fed on more frequent schedules (Tukey's post-hoc test; ANOVA, $F_{2,19} = 8.90$, $p < 0.002$; Fig. 1b).

In the field portion of the study, fish collected in July (= mid-summer) had a significantly lower $\delta^{15}N$ than fish analyzed from the other three seasons (Tukey's post-hoc test; ANOVA, $F_{3,71} = 62.02$, $p < 0.0001$; Fig. 2a), and the $\delta^{15}N$ of fish from other seasons did not differ from each other. Fish collected in July also had a significantly higher weight-to-length ratios than those from other seasons (Tukey's post-hoc test; ANOVA, $F_{3,71} = 58.55$, $p < 0.0001$; Fig. 2b).

Further analysis revealed that there was a significant overall relationship between $\delta^{15}N$ and body condition in both the laboratory ($R^2 = 53\%$, $F_{1,20} = 22.68$, $p < 0.0001$; Fig. 3) and field studies ($R^2 = 44\%$, $F_{1,73} = 57.63$, $p < 0.0001$; Fig. 4). The effect of starvation was seen both as an

overall trend, when all of the data was combined into a scatterplot, as well as within specific treatment groups. When comparing treatment groups (i.e. different feeding schedule or season collected) there was no significant difference between the slopes (ANCOVA; Lab: $F_1 = 3.60$, $p = 0.094$; Field: $F_1 = 0.64$, $p = 0.437$; Figs. 3 & 4).

Discussion

Stable isotope analysis is commonly used in food web studies to determine food sources, trophic positions, and main interactions between organisms within an ecosystem. In determining these relationships, a number of assumptions are frequently made. Data are most commonly collected in the season of highest primary and secondary production when temperatures are relatively warm in all aquatic systems and river flow rates are moderate, with a common assumption that these periods are the most relevant ecologically and are sufficiently representative of food webs in other seasons. Another common convention in stable isotope ecology is that changes in an organism's isotope ratio are assumed to reflect changes in prey type, rather than the effects of other environmental factors, including starvation or altered isotope signatures of algae and other food sources (cf., Goering et al. 1990; Hobson et al 1993; Power et al 2003; Woodland et al. 2012).

Our results demonstrate that changes in nitrogen stable isotope ratios can indicate nutritional stress as well as differences in prey consumed. In our seasonal field study and laboratory feeding experiment, we found that approximately 44% and 53% of variation in ^{15}N could be explained by a significant decrease in overall body condition resulting from reduced food consumption (as suggested by body length-weight ratios) in both field collected and laboratory raised fish, respectively (Figs. 3 & 4). Our results with fish confirm similar studies of

fasting and selective feeding in birds (Hobson et al. 1993; Kempster et al. 2007). It is not clear, however, whether different levels of nutritional stress produce the same degree of nitrogen fractionation in all species.

The variability in body condition and $\delta^{15}N$ increased when fish had access to higher resource levels (Figs. 1 & 2). In the field, this may have resulted from an increase in diet breadth. Some studies have suggested that omnivory becomes more common as food availability or ecosystem size increases (Takimoto et al 2008; Thompson & Townsend 2005; Thompson et al 2007; Williams & Martinez 2004). In our laboratory experiment, increased variance under higher resource availability may have resulted from greater physiological generalization, i.e., differential allocation of resources to metabolic processes, organ systems, or reproduction. Clearly, however, higher variance under elevated resource levels indicates that $\delta^{15}N$ isotope ratios may be altered by additional interacting factors. More laboratory experiments are needed to test fully the assumptions and limitations inherent when interpreting isotope data, as they relate not only to nutritional stress but also to changes in behavior and physiology associated with resource availability.

We recommend that investigators consider the possible effects of starvation when interpreting stable isotope data and evaluate the costs and benefits of determining body condition or nutritional stress level for their particular studies. Conclusions from our study may be particularly pertinent to studies of organisms that employ metabolic pathways that result in differing fractionation, undergo large changes in life history, experience extended periods of dormancy, or are subjected to significant variation in food availability, the latter of which may include extended periods of fasting or care of young (including nursing) that monopolizes the resources of the attending parent. By taking body condition into account, organisms may be

more appropriately placed in a food web, thereby allowing better comparisons among studies and across taxa and ecosystems.

Figures and Figure Legends

Figure 1. (a) Guppy muscle tissue $\delta^{15}N$ signature (with TetraFin Goldfish Flakes); and (b) body condition (weight-to-length) interquartile range box plots of fish fed *ad libitum* 1, 3, and 6 days. Outliers are indicated by asterisks.

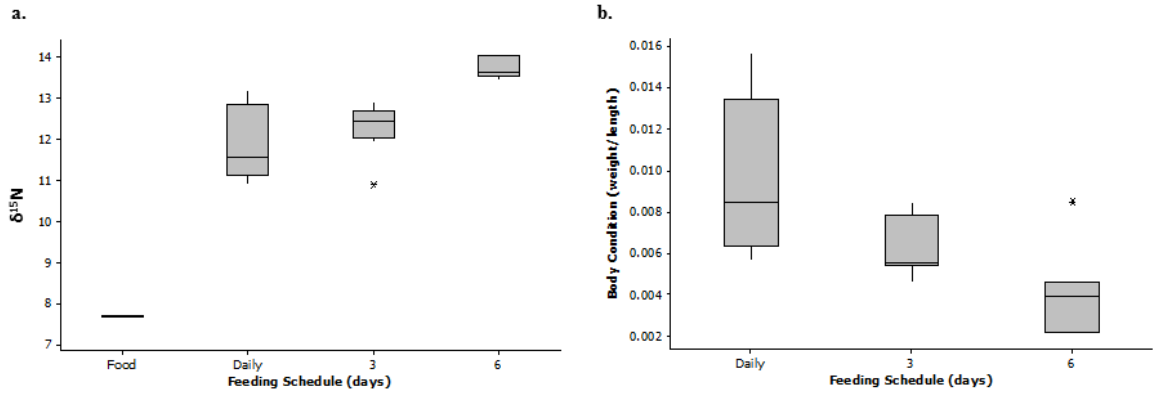

Figure 1-1 Boxplots of nitrogen isotopes and body condition from lab experiment.

Figure 2. Interquartile range box plots of: (a) $\delta^{15}N$ of muscle tissue; and (b) body condition (weight-to-length) of small fish seined from shoreline areas of the Kansas River near Lecompton, Kansas, USA during four seasons of 2013. Outliers are indicated by asterisks.

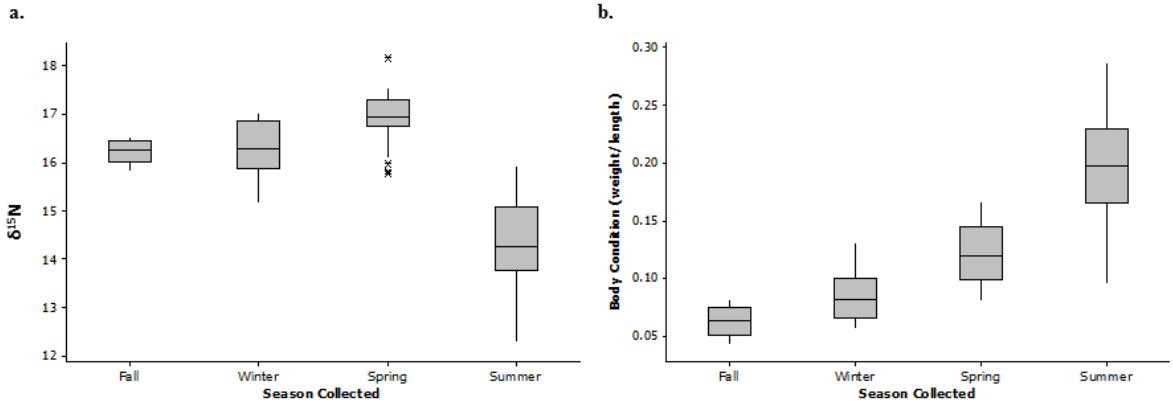

Figure 1-2 Boxplots of nitrogen isotopes and body condition from seasonal field data.

Figure 3. Inverse relationship between $\delta^{15}N$ and body condition shown as an overall trend (R^2 = 53%) as well as within individual treatments of fish fed *ad libitum* 1, 3, or 6 days.

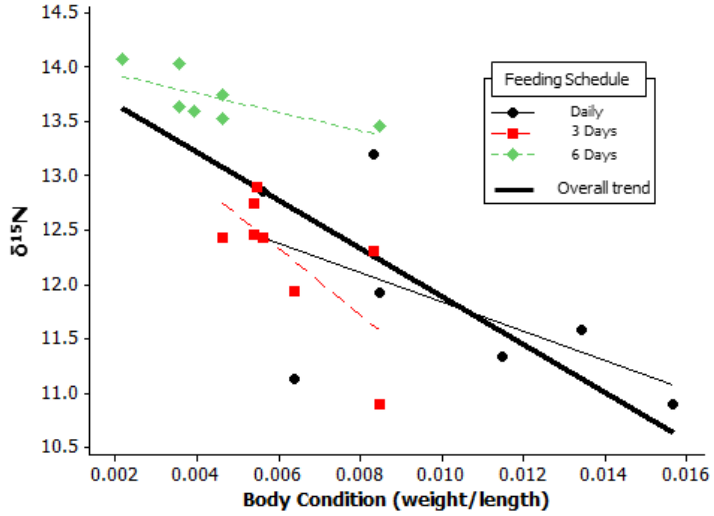

Figure 1-3 Relationship between body condition and nitrogen isotopes in lab experiment.

Figure 4. Inverse relationship between $\delta^{15}N$ and body condition observed as an overall trend (R^2 = 44%) as well as within individual seasonal collections of small fish seined from shoreline areas of the Kansas River near Lecompton, Kansas, USA in 2013.

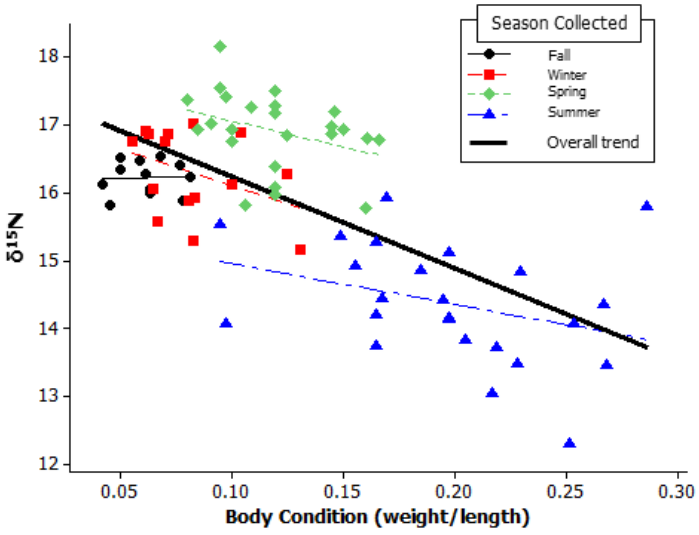

Figure 1-4 Seasonal field data relationship between body condition and nitrogen isotopes.

Chapter 2*

Consequences of employing amino acid vs bulk-tissue, stable isotope analysis:

a laboratory trophic position experiment

*Bowes, R.E. and J.H. Thorp. 2015. Consequences of employing amino acid vs bulk-tissue, stable isotope analysis: a laboratory trophic position experiment. Ecosphere, 6: art14.

Abstract

An important metric of environmental health is food web structure because it reflects species richness, natural history diversity, and resource availability. While bulk-tissue stable isotope analysis has proven valuable for food web studies, field conditions may severely restrict its use and data can be quite variable. Amino acid stable isotope analysis potentially reduces this variability, in part by eliminating the need for signatures near the trophic base because a single top consumer contains both the primary producer signature (constant phenylalanine signature) and information reflecting number of trophic transfers (a progressively increasing $\delta^{15}N$ signature of glutamic acid).

To evaluate the ecological sensitivity and cost/benefits of the techniques, we conducted a laboratory food chain experiment with four trophic levels. Water fleas (*Daphnia magna*) were cultured on a diet of powdered algae and then fed daily to guppies (*Poecilia reticulata*) for three months. These invertivorous fishes were then consumed by piscivororus bluegill sunfishes (*Lepomis macrochirus*) for a subsequent three months. All members of the food web were analyzed for ^{15}N values and degree of fractionation using both bulk-tissue and amino acid stable isotope techniques.

Our experiment demonstrated that the amino acid technique more accurately identified the true trophic position (TP) and food chain length (FCL = maximum TP) with significantly less variability around mean values for each consumer trophic level. Moreover, use of amino acids requires significantly fewer replicates to identify TP. We discuss here the relative advantages and disadvantages of both approaches for determining TP and FCL and recommend that investigators switch as soon as possible to the amino acid isotope technique for determining FCL.

Introduction

Trophic position (TP) and food chain length (FCL = maximum TP) have been assessed historically by several approaches, including behavioral observations, gut content analysis, and chemical means. While all three approaches can contribute to a better understanding of aquatic food web relationships, behavioral approaches are not feasible in the field for most aquatic invertebrates and many fish. Moreover, gut contents can be difficult to identify and count, they do not necessarily equate to assimilation, and they primarily indicate only what was ingested in the last 24 hr. Determining what organisms have assimilated over longer time scales requires chemical analyses, such as fatty acid/lipid analysis (Zelles 1999, Ruess et al. 2004, Haubert et al. 2011) and stable isotope analysis (Gannes et al., 1997, 1998, McClelland and Montoya 2002, Post 2002, Fry 2006, Popp et al. 2007, Crawford et al., 2008; Martinez del Rio et al., 2009, Chikaraishi et al. 2009, Steffan et al. 2013, Bradley et al. 2014). A major advantage of fatty acid/lipid analysis is that the investigator can often distinguish between the consumption of closely related food sources (Chamberlain et al., 2005; Ruess et al., 2004, 2005). However, the field component is challenging especially in remote areas, the laboratory methods are complex, analysis still requires information on the signature of autotrophs or basal herbivores, and the analytical costs are high. In contrast, bulk-tissue stable isotope analysis is easier to use and cheaper in many food web studies, but it cannot distinguish as well among closely related food sources.

Stable isotope analysis using bulk-tissue techniques has been a widely employed and valuable technique for analyzing TP, FCL, and other food web metrics (Gannes et al., 1997, Post, 2002, Fry 2006, Crawford et al., 2008; Layman and Post 2008, Martinez del Rio et al., 2009). For example, the ratio of heavy-to-light nitrogen ($^{15}N/^{14}N$) in tissues in comparison to an

atmospheric nitrogen standard (= $\delta^{15}N$) can be used to estimate trophic position because the value of $\delta^{15}N$ generally increases progressively up the food chain. This results because there is a tendency for selective retention of heavier isotopes and loss of lighter isotopes during physicochemical processes such as excretion, respiration, deamination, and transamination (Macko et al. 1986, 1987, Miura and Goto 2012). The $\delta^{15}N$ of a consumer has historically been considered to increase or become enriched by 3-4‰ relative to its diet (Deniro and Epstein 1981), although some studies have indicated that 1.5‰ might be a more appropriate average fractionation level (Bunn et al. 2003, Hadwen and Bunn 2005). However, the use of bulk-tissue analysis has potential limitations in aquatic systems. Field conditions often restrict its use because: (a) autotrophic sources may be unknown; (b) it is difficult to obtain clean epilithic and epiphytic algae (uncontaminated with other food items, including host vascular tissue) or suspended algae uncontaminated with dead organic matter (but see colloidal silica separation techniques in Hamilton and Lewis 1992); and (c) algal signatures are often highly variable in time and space (Herman et al. 2000, Hadwen et al. 2010, Woodland et al. 2012). In an attempt to circumvent this problem, some ecologists have used the isotopic signatures of primary (herbivorous) consumers. However, limited availability of these in some areas can pose significant problems (e.g., O'Reilly et al. 2002, Hamilton et al. 2005, Jardine et al. 2006, Wolf et al. 2009), and one never knows how representative that herbivore's diet is to the basal autotrophic source of the higher consumer. Because of this variability, investigators need to rely on large sample sizes of basal organisms and consumers to obtain a reasonable mean value.

A potential solution is to replace bulk-tissue analysis with amino acid compound specific isotope analysis (AA-CSIA). The analytical advantage is that the focal consumer contains information on both the basal signature of the primary producer and number of trophic transfers,

thereby eliminating the need for separate signatures from a primary producer. This approach works because ^{15}N isotopic signatures of some amino acids (e.g., glutamic acid) change substantially between trophic levels while others (e.g., phenylalanine) essentially remain the same (McClelland and Montoya 2002). By analyzing $\delta^{15}N$ in both glutamic acid and phenylalanine, the algal signature and number of trophic transfers (e.g., Chikaraishi et al. 2007, Popp et al. 2007, Hannides et al. 2009) are revealed. While the cost of AA-CSIA is currently much higher, due to the complexity of the analytical methods and the paucity of labs performing these analyses, this disadvantage is lessened by elimination of autotroph/basal herbivore samples and by potentially fewer samples needed at upper consumer levels.

To evaluate the relative advantages and disadvantages of these two stable isotope methods for determining TP, we first conducted a laboratory food chain experiment with four trophic levels (autotroph, herbivore, invertivore, and piscivore). Our null hypotheses were that bulk-tissue stable isotope analysis would not differ from amino acid analysis in calculated TP, variability around mean TP values, consistency of trophic fractionation, and number of replicates required for accurate prediction of TP values. Based on our results, we analyzed when and where the two techniques should be used and discussed other advantages and disadvantages of the two approaches.

Methods

Laboratory feeding experiment

We maintained all test organisms in an environmentally controlled laboratory at 21°C on a 12 h light/12 h dark cycle. Water fleas (*Daphnia magna* (Straus, 1820)) were cultured in aerated, 13-L plastic containers and fed suspended, powdered Vegetable Calcium Flakes

(Worldwide Aquatics, Inc., Arvin, CA, USA; see bestflake.com) twice weekly to apparent satiation. Guppies (*Poecilia reticulata* (Peters, 1859)) were raised through multiple generations in the lab and fed *Daphnia* daily for three months prior to being fed to a piscivorous fish (bluegill sunfish). Bluegills (*Lepomis macrochirus* (Rafinesque, 1819)) were kept individually in 95-L containers and fed one guppy per day for three months to allow for approximately complete turnover of the isotope signatures in their muscle tissues (Madigan et al. 2012). All guppies and sunfish added body weight during the experiment. This gain in mass indicates that they were replacing N isotopes during the experimental period and not starving, the latter of which would have increased their $\delta^{15}N$ values (e.g., Bowes et al. 2014).

Preparation for stable isotope analysis

All invertebrate and fish samples were washed with distilled water to remove contaminants, such as algal debris. *Daphnia* were then analyzed whole, whereas fish were dissected to isolate the muscle tissue. The tissue samples were then dried in an oven at 60°C for 48 h, ground to a fine, homogenized powder using a Wig-L-Bug® Mixer/Amalgamator (Rinn Corp./Crescent Dental Mfg. Co., Elgin, IL, USA), and held in desiccators until submitted for analysis.

Bulk-tissue stable isotope analysis

We evaluated the nitrogen isotopic composition of bulk-tissue (BT-isotope ratios) for the flake food, *Daphnia*, and muscle tissue of guppies and sunfish on half of our samples, but report here only the values for N. For this analysis we employed a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.,

Cheshire, UK) at the University of California Davis (UC-Davis) Stable Isotope Facility. BT-isotope data for each sample included total N and $\delta^{15}N$ values. The $\delta^{15}N$ values were determined from the relative difference in isotopic ratio between the samples and known standards as represented by the following equation: $\delta X = ((Rsample / Rstandard) - 1) \times 1000$, where X is ^{15}N, and the corresponding ratio is $R = {}^{15}N/{}^{14}N$. Atmospheric–nitrogen was used as the N standard. All isotope ratios are given in per mil (‰).

Amino acid stable isotope analysis

After drying, powdering, and homogenizing our samples, they were then analyzed for AA-isotope ratios at the UC-Davis Stable Isotope Facility. The general techniques for AA-isotope analysis are summarized below and described in greater detail in Walsh et al. (2014). Sample preparation involves acid hydrolysis for the liberation of amino acids from proteins and derivatization by methyl chloroformate to produce compounds amenable to GC analysis. Amino acid derivatives are injected in split (^{13}C) or splitless (^{15}N) mode and separated on an Agilent J&W factor FOUR VF-23ms column (30m X 0.25mm ID, 0.25 micron film thickness). Once separated, amino acid derivatives are quantitatively converted to CO_2 and NO_x in an oxidation reactor at 950°C, and NO_x are subsequently reduced to N_2 in a reduction reactor at 650°C. Following water removal through a nafion dryer, N_2 or CO_2 enters the IRMS. A pure reference gas (CO_2 or N_2) is used to calculate provisional δ-values of each sample peak. Next, isotopic values are adjusted to an internal standard (e.g. norleucine) of known isotopic composition. Final δ-values are obtained after adjusting the provisional values for changes in linearity and instrumental drift such that correct δ-values for laboratory standards are obtained. The $\delta^{15}N$ of two amino acids (glutamic acid and phenylalanine) were determined by this method.

Trophic position calculation

To calculate trophic position from B-T analyses, we used the following formula: Trophic Position = $[(\delta^{15}N_{consumer} - \delta^{15}N_{producer}) \div 3.4] + 1$, whereas for amino acid analyses, we employed the following modified formula: Trophic Position = $[((\delta^{15}N_{glutamic\ acid} - \delta^{15}N_{phenylalanine}) - 3.4) \div 7.6] + 1$. These two equations theoretically generate equivalent trophic position values, as illustrated in Figure 1. We employed the historically and widely used value of 3.4‰ as the denominator value in our bulk tissue trophic position estimation equation, because it yielded more accurate estimates of the known trophic position. Furthermore, it was much more accurate in our calculations than the 1.5‰ value sometimes recommended (Bunn et al. 2003, Hadwen and Bunn 2005).

Statistical analyses

Differences between bulk-tissue and amino acid stable isotope estimates of mean TPs and FCL were tested using one-way ANOVA. The variances associated with TP values generated by each isotope technique were compared with an F-test and a Levene Test. Linear regressions were run for both methods comparing actual and projected TP values, with an accurate estimation having a high R^2, a y-intercept near 0, and a slope of ~1. A power and sample size analysis was performed to find the fewest required replicates each technique required for a 95% confidence level in a trophic position calculation. The pooled standard deviation generated from the original one-way ANOVA of each technique was used as the assumed standard deviation in the power analysis for each technique (0.4906 and 0.1364 for bulk tissue and amino acid, respectively). In both cases $\alpha = 0.05$ was used along with 4 trophic positions, with a maximum difference between

trophic positions equal to 1. All data were checked for normality, unusual values, and heterogeneity of variances. All statistics were performed in Minitab 14 statistical software (Minitab Inc., State College, PA, USA) with $\alpha = 0.05$.

Results

Differences in the distribution and variability of individual amino acid analyses are evident in Figure 2, in the form of a trophic isocline graph. Trophic isoclines define the trophic position of a food web in 2-dimensional space (Chikaraishi et al. 2014). One of the advantages of this graphical method is that isotopic variability is readily perceptible (evident in the $\delta^{15}N$ values of phenylalanine along the horizontal axis). No matter what the $\delta^{15}N$ values of phenylalanine in an organism, the $\delta^{15}N$ value of glutamic acid will reflect the organism's TP. When the $TP_{Glu/Phe}$ values of organisms are displayed across trophoclines, it becomes apparent how populations simultaneously vary in trophic position and background heterogeneity of $\delta^{15}N$ values (Chikaraishi et al. 2009, 2014, Steffan et al. 2013). The $\delta^{15}N$ values of phenylalanine in a single consumer closely reflect the average of all the resources it assimilated (e.g., Chikaraishi et al. 2009, 2014, Steffan et al. 2013). This becomes important, in that the graphical representation of data points in space could reveal linear food chains within broader food web structure. All consumer species that fall within a range of $\delta^{15}N$ values for phenylalanine may effectively be using similar basal resources and fit into a distinct particular food chain, whereas a wide range of the $\delta^{15}N$ values of phenylalanine could indicate that the consumer is a generalist that can exploit resources from multiple areas or communities. Figure 2 clearly shows that the food source and three consumer species in our experiment closely aligned on their respective and distinct trophic

isoclines. Our results also show a narrow range in phenylalanine values, which in the field could indicate that the organisms were all using the same basal carbon resource.

Although both isotope techniques generated estimates of TP and FCL, their mean values were significantly different (Figs. 3 & 4). Moreover, the variance in the bulk-tissue analysis estimations of trophic position was significantly greater than that generated by amino acid analysis (Fig. 3; Table 1).

Although both techniques revealed significant differences between all trophic positions, their estimates were not equivalent. Regression analysis of TP calculated by bulk-tissue analysis compared to the expected trophic positions (= eTP; i.e., 1, 2, 3, and 4) produced the equation: $TP_{bt} = 0.514 + 0.742$ eTP, with an $R^2 = 70.0\%$. In contrast, the same regression analysis using amino acid analysis techniques yielded the equation: $TP_{aa} = -0.173 + 1.02$ eTP, with an $R^2 = 98.5\%$. AA-CSIA was, therefore, more accurate because it produced estimates with a higher R^2 value, a y-intercept nearer to 0, and a slope of approximately 1. AA-CSIA technique also produced a more accurate estimate of FCL (Fig. 4; $F_{1,16} = 16.15$, $P = 0.001$).

Statistical power analyses revealed that more samples need to be analyzed using the bulk-tissue technique compared to the amino acid isotope analysis in order to achieve 95% confidence in TP. Based on the pooled standard deviation found for each technique (Table 2; bulk-tissue = 0.4906, amino acid = 0.1364), bulk-tissue analysis required a sample size of 10 (target = 95% power, actual = 96.5%) and amino acid analysis required only 2 samples (target = 95% power, actual = 95.9%).

Discussion

Ecological and metabolic comparisons of both methods

Analysis of bulk-tissue stable isotopes is firmly established in the literature as a useful tool for exploring factors controlling food web complexity in both terrestrial and aquatic ecosystems. Laboratory analytical techniques and mathematical procedures for determining trophic position (TP) and food chain length (FCL) are well accepted (Post, 2002, Fry 2006, Crawford et al., 2008; Martinez del Rio et al., 2009). This is also a relatively inexpensive technique ($8-12 as of August 2014) and can be analyzed at many laboratories around the world fairly rapidly on both an absolute time scale and relative to the current time required for amino acid techniques. For these and other reasons, we are "not" advocating total abandonment of the BT-isotope method at this time. Nonetheless, we maintain that bulk-derived TP estimates can be profoundly inaccurate in comparison to AA-CSIA (e.g. Steffan et al. 2013) and most research questions are best tested using compound-specific stable isotope techniques, as discussed below.

Interpretation of bulk-tissue isotope data is limited by ecological conditions and metabolic processes. From an ecological perspective, both $\delta^{15}N$ and $\delta^{13}C$ can vary spatially and temporally in even pristine ecosystems — a problem which seems especially acute in rivers. Variations in flow within small to large rivers can alter $\delta^{13}C$ ratios laterally over distances of a few meters in some cases and vertically within millimeters in benthic algal layers (Herman et al. 2000, Hadwen et al. 2010, Woodland et al. 2012). Likewise, $\delta^{15}N$ ratios can vary from areas in the main channel with its mostly continuous flows to lateral backwater areas with minimal to zero flows, as both sources and N-processing pathways change (e.g., variable abundance of denitrifying bacteria) (Thorp et al. 2008). This poses significant problems for the bulk-tissue technique if, as is almost certainly the case, the predator (invertivore to top predator) is either highly mobile relative to its prey or the basal signature used in calculating TP (autotrophs or herbivore) was determined from collections made at a different time or place from the predator.

For example, ecologists typically collect grazing snails (feeding on benthic algae) and mussels (feeding on suspended algae) by hand from areas of small spatial extent but fish from large spatial extents by seining, trawling, or electroshocking from boats. If the investigator instead collects algae, the problem is magnified because the algal signature changes orders of magnitude faster than the predator's signature and over very short distances. If the investigator instead opts to use the signature from basal herbivores to circumvent this problem, he/she must first have access to longer-lived herbivores (which are difficult to find in some ecosystems) and then collect both benthic and suspension feeding herbivores to get representative ecosystem autotrophic signatures. To gain a very long perspective on changes in food webs, one can use museum samples of fish (e.g., Delong et al. 2011). The problem here, however, is that: (1) long-term algal collections are typically unavailable; (2) you can often use the external periostracum of suspension-feeding unionid mussels for a protein signature (Delong & Thorp 2009), but gaining enough periostracum from grazing snails is more problematic and museum collections of gastropods are often not as complete as for mussels; and (3) the sampled fish and herbivores were almost certainly collected by different investigators in different places and at different times (weeks to years).

These ecological problems are eliminated with AA-CSIA because the predator contains both the original basal nitrogen signature (from phenylalanine) and the trophically magnified nitrogen signatures (from glutamic acid).

From a metabolic perspective, the bulk-tissue technique suffers from at least two handicaps. First, the diet-to-tissue discrimination factors differ in both $\delta^{13}C$ and $\delta^{15}N$ among tissues in the same organism (e.g., blood, muscle, and bone) and are sometimes very large (Polito et al. 2009, Wyatt et al. 2010, Madigan et al. 2012, Xia et al. 2013). For this reason, researchers

must be consistent in choice of tissues and recognize that the isotope replacement time in a consumer from a change in diet will vary with tissue type (Sakano et al. 2005, Miller 2006, Madigan et al. 2012). While dietary change time also needs to be considered when using amino acid isotope techniques, the isotopic relationship between glutamic acid and phenylalanine stays constant in different tissues of the consumer (Chikaraishi et al. 2007, 2009). Second, the degree to which ^{15}N biomagnifies along trophic chains is poorly known for many ecological situations, and isotope signatures can fluctuate with variations in nutrient allocation within an organism, nutritional stress and body condition, and seasonal and temperature changes (Cherel et al. 2007, Kempster et al. 2007, Bowes et al. 2014). These changes will differ for each step in the food chain depending on the nutritional state of the consumer in each trophic level. As demonstrated in our laboratory experiment, the accumulated effects of metabolic variations among organisms as a whole and within tissues of a single organism will impair the investigator's ability to determine an accurate mean TP when using the number of replicates (3-5) typically employed in previous field studies. While nutritional condition affects the $\delta^{15}N$ ratio in individual amino acids, the effects are comparable among similar amino acids, and thus the nitrogen relationship between glutamic acid and phenylalanine is unchanged.

Statistical comparisons

Our laboratory experiment showed that the amino acid stable isotope technique was a substantial improvement over the bulk-tissue technique based on statistically significant differences in the calculations of TP and FCL. In comparison to the bulk-tissue calculations, the amino acid stable isotope technique more accurately calculated TP values for each trophic level from herbivore through piscivore. Moreover, our analyses of power and sample size (using the

pooled standard deviation for each technique) found that to obtain 95% confidence in calculations of TP and FCL, an investigator using the bulk-tissue technique would require data from at least 10 consumers, whereas someone using the amino acid technique would need only 2 (but we suggest a minimum of 3 to obtain a statistical mean).

Alternative choices

If financial costs and laboratory time were not an issue is selection of isotope technique, the clear choice of analytical method would be AA-CSIA. As described above, it provides more accurate and precise estimates of trophic position and food chain length with fewer required sample replicates. Furthermore, the methods and time to process tissue samples in the field are the same with the two methods, but the overall time in the field is reduced with the amino acid method because you need to collect only the target consumers (and fewer of those) rather than those consumers plus either autotrophs or basal herbivores (assuming they are even easily available).

The main disadvantages of AA-CSIA at this time are: (a) the laboratory analytical methods are complex and the methods employed may not yet be consistent among laboratories; (b) because of this complexity, processing time and analysis turnaround is longer than the simpler and more traditional methods used in bulk-tissue analysis; (c) fewer isotope laboratories around the world offer such analyses, thereby increasing analytical time; and (d) the cost of the analysis is much greater than bulk tissue analysis. In the latter instance, the best of a wide range of analytical prices we found in the USA in late 2013 was $65 plus shipping for one isotope and $97 for two (based on complete chemical processing/analysis of weighed and dried tissue). However, each analysis includes values for 12 or more individual amino acids.

While a cursory look at the prices cited above might lead one to automatically choose the bulk-tissue approach in trophic position studies if cost are a major concern, the choice is in fact more complex for financial as well as scientific reasons. In predicting expenditures, the investigator needs to account for the greater field costs (personnel, equipment, and time) and analytical fees from collecting basal organism signatures and extra consumer replicates. This assumes also that the autotrophs or substitute herbivores are available and representative of what ultimately ends up in the tissue of the higher consumers. If the study's focus is on food sources and/or food web complexity, then the bulk-tissue method requires collection of a large number of potential terrestrial and aquatic sources of whole, particulate, and dissolved organic sources from local and areas upstream areas. The cost for this does not rise on a per sample basis in B-T analysis because most labs provide isotope values for both C and N. In contrast, the costs rise by about 50% when analyzing two isotopes with AA-isotope procedures. However, the output from AA-isotope analysis includes a dozen or more amino acids, thereby allowing the investigator to more precisely identify food sources.

Based on our experimental results, we recommend that investigators switch as soon as they can from the bulk-tissue to the amino acid stable isotope technique to gain a much more accurate analysis of food chain length. This recommendation is consistent with recent conclusions of other scientists (Gannes et al. 1997, Martinez del Rio et al. 2009, Wolf et al. 2009). Although the relatively greater accuracy for evaluating food sources of carbon AA-isotope techniques over BT-isotopes is still waiting sufficient experimental confirmation, we strongly suspect that this technique will also prove superior to traditional approaches.

Figures and Figure Legends

Figure 1. (a) Trophic position is calculated from bulk-tissue stable isotope analysis using the equation: TP = [($\delta^{15}N_{consumer}$ - $\delta^{15}N_{producer}$) ÷ 3.4] + 1. (b) Trophic position using amino acid compound specific isotope analysis is calculated using the equation: TP = [(($\delta^{15}N_{glutamic\ acid}$ - $\delta^{15}N_{phenylalanine}$) – 3.4) ÷ 7.6] + 1.

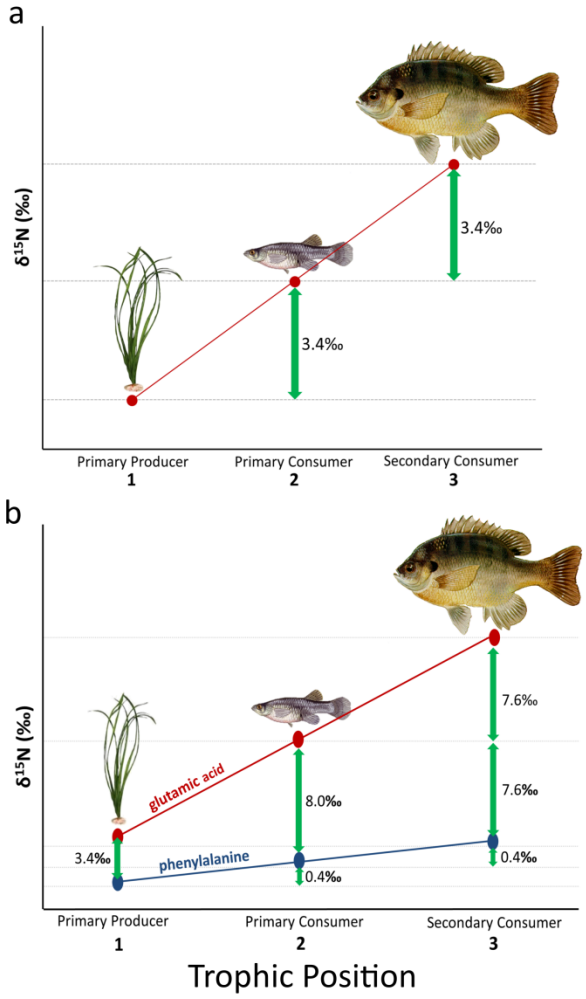

Figure 2-1 Trophic position calculations.

Figure 2. Cross plot for $\delta^{15}N$ values of glutamic acid and phenylalanine. Trophic isoclines are created for each trophic position using the equation $TP = [((\delta^{15}N_{glutamic\ acid} - \delta^{15}N_{phenylalanine}) - 3.4) \div 7.6] + 1$, each with a slope of 1.0 and between-line interval of 7.6.

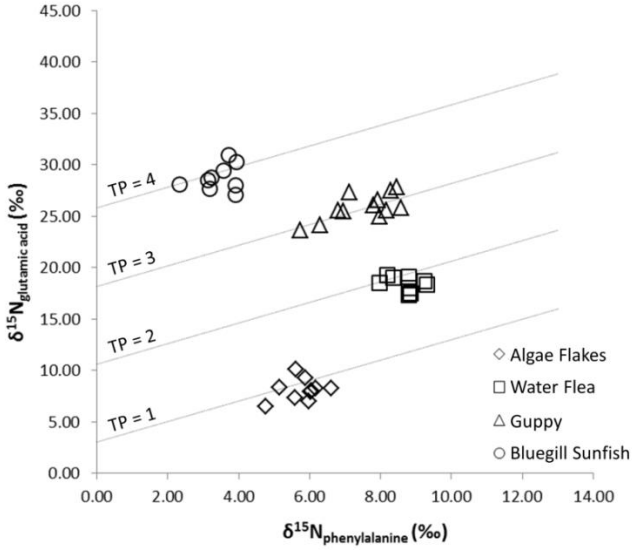

Figure 2-2 Cross plot of lab feeding experiment.

Figure 3. Interquartile range box plots of calculated trophic position of each feeding group using (a) bulk-tissue stable isotope analysis; and (b) amino acid compound specific isotope analysis.

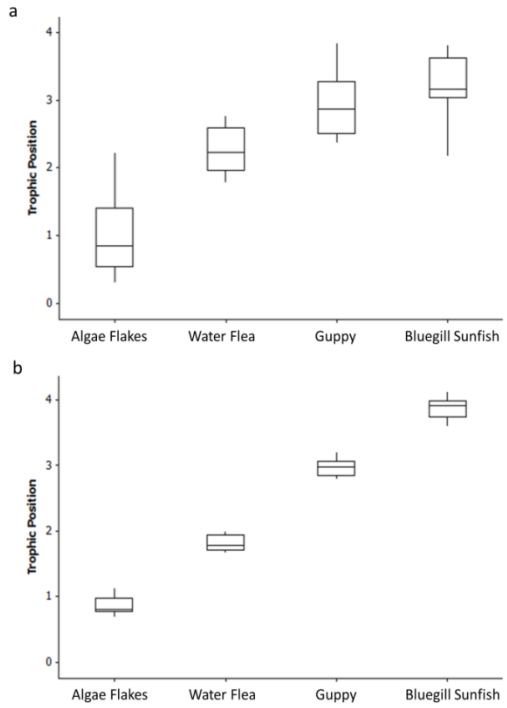

Figure 2-3 Trophic position boxplots.

Figure 4. Interquartile range box plot of the estimation of food chain length using the two techniques. There is a significant difference between the two technique's calculation of food chain length ($F_{1,16}$ = 16.15, P = 0.001). Amino acid analysis' estimation of food chain length is closer to the actual known food chain length of 4 for this laboratory experiment.

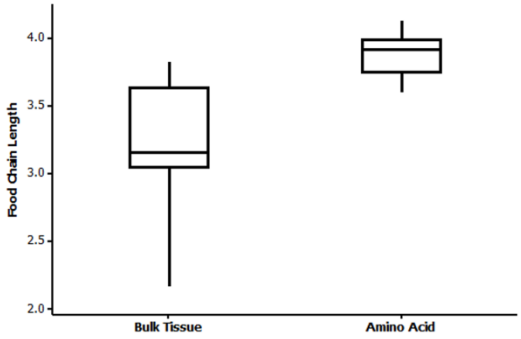

Figure 2-4 Food chain length estimates.

Tables

Table 1. Test for equal variances between bulk-tissue and amino acid stable isotope analysis within each feeding group.

		F test		Levene's Test	
Organism	Sample size	Test Statistic	P value	Test Statistic	P value
Algae flakes	10	0.05	0.000	5.15	0.036
Water flea	9	0.12	0.007	10.06	0.006
Guppy	12	0.07	0.000	9.98	0.005
Bluegill sunfish	9	0.11	0.005	2.85	0.111

Table 2-1 Test of equal variances.

Table 2. Trophic position estimates for each feeding group using both bulk-tissue stable isotope analysis and amino acid compound specific isotope analysis. There were significant differences between all trophic positions using both techniques.

			ANOVA			
Bulk-Tissue Analysis	Mean	Standard Deviation	$F_{3,36}$	P	R^2	Pooled SD
Algae flakes	1.0000	0.5957	40.33	0.000	77.07%	0.4906
Water flea	2.2844	0.3555				
Guppy	2.9550	0.4843				
Bluegill sunfish	3.1989	0.4849				
Amino acid Analysis						
Algae flakes	0.8542	0.1373	904.68	0.000	98.69%	0.1364
Water flea	1.8292	0.1234				
Guppy	2.9691	0.1253				
Bluegill sunfish	3.8830	0.1603				

Table 2-2 Trophic position estimates.

Chapter 3

Multidimensional metrics of niche space for use with various analytical techniques

Abstract

Multidimensional data are integral to many community-ecological studies and come in various forms, such as stable isotopes, compound specific analyses (e.g., amino acids and fatty acids), and both biodiversity and life history traits. Scientists employing such data usually lack standardized metrics to evaluate communities in niche space where more than 2 dimensions are involved. To alleviate this problem, we developed a graphing and analytical approach for use with more than two variables, based on stable isotope bi-plot metrics first presented by Layman et al. (2007). We introduce here our community metrics as R scripts. By extending the original metrics to multiple dimensions and offering alternative versions using Manhattan distance instead of Euclidean distance, we created n-dimensional plots and metrics to characterize any set of quantitative measurements of a community. We demonstrate here the utility of these newly-developed metrics using stable isotope data; however, the approaches are widely applicable to many types of data. The resulting metrics provide more and better information compared to traditional analytic frameworks. The approach can be readily applied in many branches of community ecology, and it offers accessible metrics, readily comparable across communities, to quantitatively analyze the structure of communities across ecosystems and through time.

Introduction

Multivariate data are necessary to understand ecological community structure, as species assemblage is an integrative process responsive to a complex set of biotic players and environmental drivers. While the ability to understand interactions among community components has benefitted from the development of several new analytical technologies, these developments have been rapid, and therefore there is a need for ever improving mathematical models and metrics to allow researchers to cope with new data and compare communities among systems and through time.

Many studies in community ecology acquire data from analysis of either bulk-tissue stable isotopes (e.g., Peterson & Fry 1987, Fry 2006), compound-specific stable isotopes (as from amino acids [e.g., Fogel & Tuross 2003, Walsh et al. 2014]), fatty acid analysis (e.g., Ackman & Eaton 1966, Hammer et al. 1998, Iverson et al. 2004), or the analysis of biodiversity or life history traits (e.g., Grime 1977, Southwood 1977, Kearney & Porter 2009). Below we briefly describe the scientific fields where access to multidimensional metrics, such as those offered in this paper, would enhance our knowledge of natural populations, communities, and ecosystems.

The method of stable isotopes has expanded tremendously in its areas of application from its earliest uses. Natural abundance isotope signatures can be used to find patterns and mechanisms at the single organism level, assess the structure and dynamics of food webs, and trace the origins and migrations of species across the globe (Fry 2006, Hobson & Wassenaar 2008). Isotopes have also been employed to follow whole ecosystem nutrient cycling in both terrestrial and marine systems and to examine global element cycles, past climatic conditions, hydrothermal vent systems, and rock sources (Robinson 2001, SCOR Working Group 2007,

Michener & Lajtha 2008). As a consequence, isotopic analysis has almost become a standard "instrument" in the toolbox of many physiologists, ecologists, geochemists, and scientists studying element or material cycling in the environment. Many ecologists employ stable isotopes in food-web studies, using nitrogen (N), carbon (C), sulfur (S), oxygen (O), and/or hydrogen (H). $\delta^{15}N$ (ratio of nitrogen isotopes, ^{15}N to ^{14}N, expressed relative to a standard) enriches stepwise with trophic transfers of biomass and is the dominant tool for estimating trophic position of organisms (Minagawa & Wada 1984, Peterson & Fry 1987, Post 2002). $\delta^{13}C$ (ratio of carbon isotopes, ^{13}C to ^{12}C, expressed relative to a standard) can be used to determine original sources of dietary carbon, because it varies substantially among primary producers with different photosynthetic pathways (e.g. C_3 *vs.* C_4 photosynthetic pathways), and changes little with progression through a food web (DeNiro & Epstein 1981, Inger & Bearhop 2008, Peterson & Fry 1987, Post 2002). Similarly, the ratio of sulfur isotopes ($\delta^{34}S$) varies markedly among primary producers but changes relatively little with trophic transfers, and can also be used to identify important basal resources, especially in marine systems (Currin et al. 1995, Peterson & Howarth 1987, Jones *et al.* 2010). The $\delta^{18}O$ and $\delta^{2}H$ values of groundwater and precipitation vary at multiple spatial scales, allowing researchers to decipher patterns across small-scale environmental gradients (Deines et al. 2009, Finlay et al. 2010, Solomon *et al.* 2009, 2011) or decode large-scale dietary patterns across geographic regions (Bowen & Revenaugh 2003, Bowen et al. 2005, Vander-Zanden et al. 2016). Most frequently, these elemental tracers are plotted and analyzed two at a time, in a bivariate approach. Here, we offer meaningful metrics that can accommodate more axes and allow researchers to explore a greater number of niche dimensions simultaneously.

Aside from the plethora of natural abundance isotopes that can be measured, ecologists now also have the ability to detect the isotopic signatures of individual compounds occurring within bulk tissue, such as fatty acids, amino acids, and biomarkers (Lichtfouse 2000, Krummen et al. 2004, Sessions 2006). Fatty acids represent a diverse group of molecules that comprise the majority of lipids in all organisms. Because of their biochemical restrictions and unique origins in plants and animals in some cases, fatty acids have proven to be a powerful tool in delineating food webs and assessing predator diets (e.g., Ackman & Eaton 1966, Hammer et al. 1998, Iverson et al. 2004, Budge et al. 2006, Morrison et al. 2010). Similar to fatty acids, amino acids are biologically important compounds; they are the dominant nitrogen-bearing biomolecules of organisms and are the structural monomers that make up proteins. Patterns of isotopic fractionation during synthesis and transamination of amino acids can be used to determine trophic linkages, follow nutrient pathways, and distinguish between primary production sources (e.g., Fantle et al. 1999, Fogel & Tuross 2003, Larsen et al. 2009, Chikaraishi et al. 2009, Walsh et al. 2014, Bowes & Thorp 2015). Biomarkers are compounds that are produced by only a limited group of organisms. The use of biomarkers in ecological studies has seen a rise in recent years, particularly in the field of microbial ecology, which uses a combination of deliberately added tracers and isotopic analysis of biomarkers to directly link microbial identity (as assayed with the biomarker), biomass (the concentration of the biomarker in the habitat medium), and activity (isotope assimilation) (Boschker & Middelburg 2002, Peters et al. 2007). All these approaches have led to accumulation of multidimensional data.

Traits-based approaches have a long history in community ecology (e.g., Grime 1977, Southwood 1977, Connell 1978, Keddy 1992, Weiher & Keddy 1995). Traits have been used in conceptual models to describe qualitatively the life history traits of organisms occurring in a

community (Grime 1977, Southwood 1977, Connell 1978) and quantitatively to model trait–environment relations as a means of predicting species presence/absence (e.g., Keddy 1992, Weiher & Keddy 1995) or abundance (e.g., Chesson et al. 2002). Traits have recently been employed to estimate community and ecosystem responses to rapid environmental change, including climate drivers (e.g., Thuiller et al. 2007, Morin & Lechowicz 2008). The potential of traits-based approaches has motivated reanalysis of existing trait datasets and acquisition of new data to test many relevant ecological questions (e.g., Kolar & Lodge 2001, Norberg et al. 2001, Chesson et al. 2002, Loreau et al. 2003, Naeem & Wright 2003, Cornwell et al. 2006, McGill et al. 2006, Olden et al. 2006, Shipley et al. 2006, Savage et al. 2007, Cornwell & Ackerly 2009, Kearney & Porter 2009). There has been a rapid increase in the application of traits-based approaches, and many associated conceptual advances in analyzing complex trait data have been made (e.g., Webb et al. 2010). With all this progress, the need to quantitatively compare communities in multidimensional trait-space is higher than ever.

Whichever form of multidimensional data researchers employ, they need easily accessible, widely applicable, standardized, and broadly comparable metrics to quantitatively analyze community structure in the given space. Numerous hypothesis-testing frameworks and analytical approaches have been proposed to characterize two-dimensional data (Wantzen et al. 2002, Layman et al. 2007, Newsome et al. 2007, Schmidt et al. 2007, Turner et al. 2010). Of those that relate to niche metrics, one of the most commonly used was developed by Layman et al. (2007, 2012). It has proven useful in measuring dispersion of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in bivariate space (i.e. Laymen et al 2012). Goals of this paper include extending the Layman metrics to multiple dimensions, proposing modifications that are useful in a multidimensional setting, and illustrating the use of the extended metrics.

To enhance the ability of researchers to analyze data in three or more dimensions, we introduce here community metrics as R scripts. By extending the original Layman et al. (2007) metrics (which use the standard Euclidean distance in trait space) to multiple dimensions and also offering alternative calculations making use of Manhattan distance, we create n-dimensional plots and metrics useful for characterizing quantitatively set of quantitative measurements of a community. Metric values we include are: Ranges for each variable considered (i.e. $\delta^{13}C$ range or $\delta^{15}N$ range if $\delta^{13}C$ and $\delta^{15}N$ are two variables measured for species in a community); mean distance to the centroid of points in multidimensional space (CD), which acts as a measure of species spread; mean nearest neighbor distance in multidimensional space (NDD), functioning as a measure of density of species packing; standard deviation of nearest neighbor distance (SDNND), measuring evenness of species packing in n-dimensional space; and total convex hull area (2 dimensions) or volume (more than 2 dimensions; CHV), acting as a measure of the total amount of niche space occupied by the community. We demonstrate the utility and ecological meaning of these newly-developed multidimensional metrics using stable isotopes; however, the approach is widely applicable to many other types of data.

Methods

Metrics

Range (R): Total distance between the farthest separated species in that axis (i.e. maximum – minimum). The examples in this manuscript use stable isotope analyses of $\delta^{13}C$ and $\delta^{15}N$, as was first proposed by Layman et al. 2007. $\delta^{13}C$ isotope signatures are generally used to investigate basal, organic matter sources supporting an organism, and $\delta^{15}N$ signatures are used to determine trophic position. Mean distance to the centroid (CD): Average Euclidean or Manhattan

distance of each species to the centroid, where the centroid is the mean value of each axis for all species. The CD metric functions as a measure of species spread. Mean nearest neighbor distance (NND): Mean of the Euclidean or Manhattan distances to each species' nearest neighbor in n-dimensional space. The NND metric functions as a measure of density of species packing. Standard deviation to the nearest neighbor (SDNND): The standard deviation of the mean nearest neighbor distance in n-dimensional space, using either Euclidean or Manhattan geometry. The SDNND metrics acts as a measure of evenness of species packing in n-dimensional space. Convex hull volume (CHV): Convex hull area (2 dimensions) or volume (more than 2 dimensions), is the volume encompassed by all species in the n-dimensional space. The CHV represents a measure of the total amount of niche space occupied by the community. The R script (version 3.2.1) to calculate all of these metrics in both Euclidean and Manhattan distance is included in the supplementary material (Appendix 1). Also included with the calculations for the metric values is a script to create confidence intervals around each metric value. This involves a resampling protocol, where the researcher can define the number of resampling events, and create 99% or 95%. These can be created with individual-level data, where the code resamples individuals within a species, if there are at least 2 or more individuals of a species. The confidence intervals can also be created using species-level data with associated standard errors. This resamples values from a normal distribution with the given standard error but is subject to researcher defined bounds.

Decomposition possibilities of our community metrics with Manhattan distance

These community metric values can be calculated in the originally offered Euclidean distance (Layman et al. 2007), or as an alternative can be now calculated in Manhattan distance

(Fig. 1). When using the metric values calculated in Manhattan distance, it is possible to normalize and see how much more info you gain from each new dimension, as a way to assess the extent to which the additional isotope data clarify niche dimensionality. This is most easily demonstrated in the CD metric, as seen in Equation 1. This then gives you a value of how much variability or information is coming from each dimension (one of the axes measured by Manhattan distance (Fig. 1)).

$$\frac{CD_{Man}}{N} = \frac{1}{SN}\sum_{i=1}^{S}\left\|x^{(i)} - c\right\|_1 \quad (1)$$

Museum fish samples for the Lower Ohio River

Museum collections and species surveys by government agencies provide data potentially useful for analyzing long-term environmental impacts (Vander Zanden et al. 2003, Gido et al. 2010) as well as spatially dispersed ecological processes. We analyzed food sources and trophic position of piscivorous and invertivorous fishes from the Lower Ohio River (Evansville, Indiana to Cairo, Illinois USA) using preserved specimens from museums. Samples were donated by the Bell Museum, Field Museum, Illinois Natural History Survey, Illinois State Museum, Milwaukee Public Museum, Ohio State University Museum of Biological Diversity, Southern Illinois University, University of Michigan Museum of Zoology, and University of Wisconsin - Stevens Point. The largest preserved specimens were chosen for tissue harvesting; however, museum specimens of fish tend to be small in general, reflecting the need to conserve limited shelf space.

Sample processing and isotope analysis of fish tissue from the Lower Ohio River

We extracted muscle tissues from an area between the lateral line and dorsal fin of adult fish preserved in today's museums in ethyl alcohol and probably previously for short or long periods in formalin. Neither preservative significantly alters the isotopic results (Hannides et al. 2009, González-Bergonzoni et al. 2014). Tissue samples were rinsed with deionized water, placed in pre-combusted glass vials, dried at 60°C for 48 hr, and then ground into a fine, homogenized powder using a Wig-L-Bug® Mixer/Amalgamator.

After samples were dried, powdered, and homogenized, their $\delta^{13}C$ and $\delta^{15}N$ bulk tissue and amino acid stable isotope ratios were determined at the UC-Davis Stable Isotope Facility. The data for each bulk tissue sample included total N and C and $\delta^{13}C$ and $\delta^{15}N$ values. The $\delta^{13}C$ and $\delta^{15}N$ values were determined based on the relative difference in isotopic ratio between the samples and known standards, as represented by the following equation: $\delta X = ((R_{sample} / R_{standard}) - 1) \times 1000$ where X is ^{15}N, the corresponding ratio is $R = {}^{13}C/{}^{12}C$ or $R = {}^{15}N/{}^{14}N$. Vienna Pee Dee Belemnite is used as the standard ratio for carbon and atmospheric nitrogen was used as the N standard. All isotope ratios are given in per mil (‰).

General techniques for compound specific isotope analysis of amino acids (AA-CSIA) are summarized below and extensively described in Walsh et al. (2014). Sample preparation involves acid hydrolysis for the liberation of amino acids from proteins and derivatization by methyl chloroformate to produce compounds amenable to GC analysis. Amino acid derivatives are injected in split (^{13}C) or splitless (^{15}N) mode and separated on an Agilent J&W factor FOUR VF-23ms column (30m X 0.25mm ID, 0.25 micron film thickness). Once separated, amino acid derivatives are quantitatively converted to CO_2 and NO_x in an oxidation reactor at 950°C, and NO_x are subsequently reduced to N_2 in a reduction reactor at 650°C. Following water removal through a nafion dryer, N_2 or CO_2 enters the IRMS. A pure reference gas (CO_2 or N_2) is used to

calculate provisional δ-values of each sample peak. Next, isotopic values are adjusted to an internal standard (e.g. norleucine) of known isotopic composition. Final δ-values are obtained after adjusting the provisional values for changes in linearity and instrumental drift such that correct δ-values for laboratory standards are obtained. Signatures of both $\delta^{13}C$ and $\delta^{15}N$ were determined for the following amino acids and expressed as per mil (‰): Alanine, Aspartic Acid, Glutamic Acid, Glycine, Isoleucine, Lysine, Methionine, Phenylalanine, Proline, Tyrosine, and Valine. Tyrosine signatures were excluded from analyses dues to missing measurements caused by concentrations below detection limits.

Trophic position and food source calculations using amino acids

To calculate trophic position of consumers from AA-CSIA data, we employed the following formula: TP = [(($\delta^{15}N$ of Glutamic Acid - $\delta^{15}N$ of Phenylalanine) - 3.4) ÷ 7.6] + 1 (e.g. Chikaraishi et al. 2007, 2009, 2014, Popp et al. 2007, Hannides et al. 2009, Steffan et al. 2013, Bowes & Thorp 2015).

To calculate the amino acid composition of food sources, we measured isotopic signatures using $\delta^{13}C$ AA-CSIA for three replicates of the following potential aquatic and terrestrial food sources, as represented biochemically by: cyanobacteria (*Spirulina*), green algae (*Chlorella sp.*), fungi (baker's yeast or *Saccharomyces cerevisiae*), C_3 terrestrial plants (C_3 grasses (*Elymus sp*., probably *E. virginicus*), C_3 tree leaves (cottonwood, *Populus deltoides*), two C_3 crops (wheat, *Triticum aestivum*, and soybean, *Glycine max*)), a C_4 terrestrial plant (corn, *Zea mays*), and a C_3 aquatic vascular macrophyte (wild celery, *Valisneria americana*). These specific food sources were chosen as they represent common food sources available in rivers across the US. The terrestrial sources were collected in Lawrence, Kansas, and aquatic sources were

ordered from laboratory cultures (PureBulk.com). These new signatures were used in conjunction with data from other aquatic studies (Larsen et al. 2009, Larsen et al. 2013) to determine classification and specific isotopic fingerprints of the different food sources.

$\delta^{13}C$ values of each of the amino acid were normalized to their respective sample means ($\delta^{13}C_{AA}$ – mean $\delta^{13}C_{AA}$), and tested for univariate normality. Normalizing the values to the means removes any effect of growth media between the different food sources. To explore patterns and determine producer food groups we performed principle component analysis on normalized $\delta^{13}C$ signatures of all available amino acids, this showed that samples clustered according to major phylogenetic associations (6 major groups were identified: cyanobacteria, algae, fungi, C_3 terrestrial, C_4 terrestrial, and aquatic macrophyte). Differences in each amino acid $\delta^{13}C$ signatures between these different producer groups were tested with ANOVA. Then we performed linear discriminant function analysis on $\delta^{13}C$ AA-CSIA to determine the combination of $\delta^{13}C$ AA-CSIA values (independent variables, in this case 9 amino acids: Alanine, Aspartic Acid, Glutamic Acid, Glycine, Isoleucine, Lysine, Phenylalanine, Proline, and Valine) that best explained differences between food sources (categorical variables determined by principle component analysis), and we used a leave-one-out cross validation approach to calculate the probability of food source group membership of the classifier samples. To test that there were no difference in classification between the groups Pillai-Bartlett trace (MANOVA) was applied. All preliminary analyses on food sources were done in Minitab 14 (Minitab Inc., State College, PA, USA), and can be found in Chapter 5 of this Dissertation.

Relative contributions of dietary amino acids to consumers were estimated using the software "Food Reconstruction Using Isotopic Transferred Signals" (or FRUITS; Fernandes et al. 2014, 2015). Normalized $\delta^{13}C$ values as well as their associated uncertainties (±1 S.D.), for

each consumer species and potential food sources in the river was input into the FRUITS model. FRUITS incorporates the capability to account for dietary routing; that is, the contribution of different original primary production sources towards the amino acids signals measured in the consumer. It was assumed that all food sources were equally likely and had the potential to make up 100% of the diet of the consumer. No other priors were used in the model. FRUITS is executed with a software package for performing "Bayesian inference Using Gibbs Sampling" (or BUGS), and also considers the biochemical composition of sources and which sources are most likely to contribute the most (see http://www.mrc-bsu.cam.ac.uk/software/bugs/). The FRUITS output is a summary of percent contributions of each potential food source to the consumer's diet along with standard deviation and confidence intervals. FRUITS version 2.0 (http://sourceforge.net/projects/fruits/) was used for estimating food source contributions. Taking into account posterior uncertainties in the proportional contributions of different food sources and food source combinations, sensitivity analyses were conducted to evaluate the reliability of the results (Fernandez et al. 2014).

Results

Analyzing data in more than two dimensions provides many benefits, as demonstrated here for analysis of stable isotope data using our community metrics. Note, however, that these metrics could be used seamlessly on any set of quantitative measurements of aspects of a community (e.g., fatty acids, amino acids, traits). We use stable isotope data here because they are employed widely to assess the structure and dynamics of food webs; and because many ecologists already analyze their data with two-dimensional Layman metrics, but the larger numbers of both bulk-tissue and fatty- and amino- acid stable isotope ratios that are increasingly

used indicate the need for multivariate approaches. There are numerous hypothesis-testing frameworks and analytical approaches that have been proposed to characterize dispersion of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in bivariate space; however, the need for analytical tools in more dimensions is becoming increasingly evident. To illustrate the need for, and value of multidimensional metrics, we illustrate this below for various conceptual, ecological situations and an actual long-term museum data set.

The first schematic (Fig. 2) shows how the metrics of two communities that are nearly identical in two dimensions could be influenced by a third dimension. The two theoretical communities look similar in two dimensions (Fig.2a–b; when only using $\delta^{15}N$ and $\delta^{13}C$) and all metric values are nearly identical. When more information is added as a third dimension (Fig. 2c–h; for example another isotope, $\delta^{\#}I$), it may be revealed in a variety of possible ways that the communities really differ. The additional dimension could vary little (Fig. 2c) or widely (Fig. 2d). The difference in range for the new dimension, IR, distinguishes these cases, and also affects the other metrics (metrics are much greater in 2d than 2c). Alternatively, all species could be similar to each other except for one taxon (Fig. 2e), or all could vary widely (Fig. 2f). In Fig. 2e, f, the ranges the third dimension are the same in both communities, but other metric values differ, reflecting the difference between the communities. Lastly, the additional dimension could be highly correlated with one of the previous dimensions (Fig. 2g), or may be uncorrelated (Fig. 2h). In the correlated case, metric values are similar to the two dimensional measures (Fig. 2a), with little new information gained from the new dimension.

The second schematic demonstrates how the metrics of a community could be affected by the addition of three new species in two and three dimensions. Figure 3 presents a community of organisms prior to the addition of any new species in two (Fig. 3a) and three (Fig. 3b)

dimensions. In the 3D example, all species are similar with respect to their third dimension ($\delta^{\#}I$) values, with IR being very small (Fig. 3b). Then, three examples are offered to illustrate how three new species introduced into an existing community could alter community metrics if only two dimensions of isotopes are measured (Fig. 3c–e). The new species could differ from the original community in either the vertical dimension (Fig. 3c; in this example $\delta^{15}N$) or in the horizontal dimension (Fig. 3d; in this example $\delta^{13}C$). The addition of these new species would then be reflected strongly in changes in the two dimensional metric values (Fig. 3 c–d). However, if the new species did not differ markedly from the previous community in the two measured dimensions (Fig. 3e), changes to the community would be difficult to detect using only two dimensional metrics. However, the new species of 3e could be revealed to differ strongly from the rest of the community when a third isotope ratio is measured (Fig. 3 g, h). The new species could be similar to each other and differ from the existing community with regard to the third dimension (Fig. 3g). Aside from the obvious increase in the range of the third dimensions (IR) from Fig. 3 b to g, CD and CHV also increase. The new species could also differ not only from the existing community, but also from each other with respect to the third dimension (Fig. 3h). In this case, IR, CD, NND, CDNND, and CHV all increase in response to the new community members (Fig. 3b to h). CD and CHV do not increase as dramatically from b to h as they do from b to g. The new species of 3e could, alternatively, be similar to the previous community with respect to the third dimension (Fig. 3f; $\delta^{\#}I$) as well as with respect to the first two dimensions, showing little change in 3D as well as 2D metric values before and after invasion. In that case, additional isotope dimensions beyond the third may yet reveal community effects of the added species.

Finally, we demonstrate the utility of the metrics with real data from compound-specific stable isotope analysis of amino acids ($\delta^{13}C$ and $\delta^{15}N$) from fish collected before and after dam construction on the Lower Ohio River (Fig. 4). In two dimensions, the communities look similar before (a) and after (b) dam construction, and metric values are similar. The addition of a third dimension either alters apparent relationships among fish species or has little effect on our perception of the communities, depending on which amino acid is used and reflecting the fact that some dimensions can contain more information about trophic diversity that others, depending on the community. Relationships among species seemed to change very little from before (Fig. 4c) to after (Fig. 4d) dam construction when we added the $\delta^{13}C$ signature for glutamic acid (isotopic range from -35 to -15). In contrast, substantial differences (but not significantly so, as confirmed by resampling and 95% confidence intervals) were evident in community relationships from before (Fig. 4e) to after (Fig. 4f) when using the $\delta^{13}C$ signature for lysine (essential amino acid).

The metric values in the conceptual Figures 2 and 3 and the real data in Figure 4 and 5 were developed using Euclidean geometry. All the metrics are also available to users based on Manhattan distances (for difference between Euclidean and Manhattan distances, see Fig. 1). There are several benefits to using Manhattan distance, as discussed in the Methods section. The decision of which metric geometry to use is at the discretion of each researcher, and both could be used when presenting results. For example, the Manhattan distance could be employed to determine the amount of new information gained from each additional dimension. Through decomposition of the Manhattan metrics, like that of CD, we can determine the amount of new information gained by each of the additional amino acid signature in Figure 4. Based on CD, the additional information gained by glutamic acid is lower (2.0) than the amount gained by looking

at lysine (3.97), however both provide at least double the amount of variability, and in turn information on how the communities differ, than bulk tissue isotope values alone (1.90).

We can delve even deeper into what the new amino acid isotope values and metrics mean in terms of community structure before and after dam construction by calculating fish trophic positions and the proportion of different food sources in fish diets (Fig. 5). Compound specific carbon isotope values of amino acids and a Bayesian mixing model was used to compute the percentages of algae, C_3 terrestrial plants, C_4 terrestrial plants, aquatic macrophytes, and cyanobacteria in the fish species' diets. Trophic position was calculated with compound specific nitrogen isotope values of phenylalanine and glutamic acid using a trophic position equation (Chikaraishi et al. 2007, 2009, 2014, Bowes & Thorp 2015). Metric values were then calculated using all 6 of these new dimensions (though only three dimensions could be plotted). This allows us to show the utility of combining plots with the use of the metric calculations to tease apart community structural differences. Before dam construction (Fig. 5a), one species (Bluecat) differed substantially from the others. After dam construction (Fig. 5b), however, many of the ranges shifted and CD, NND, SDNND, and CHV all decreased. Moreover, species specific shifts in diet and trophic position were evident, e.g., bluecat clustered with the other species. We were able to determine that the differences between SDNND and CHV before and after dam construction were significant due to non-overlapping 95% confidence intervals created using a resampling protocol.

Discussion

Both resulting Euclidean and Manhattan community metrics provide increased resolution and reveal the influence of new dimensions on community structure as compared to traditional

analytical frameworks. Higher metric values indicate more spread in the overall community structure, with each metric value reflecting a different measure of spread. CD shows species spread in n-dimensional space, NND indicates density of species packing, SDNND corresponds with the evenness of species packing, and CHV reflects the total amount of niche space occupied by the community.

As seen in Figure 2, the addition of a third dimension has the potential to reveal a great deal of information pertinent to community structure. The additional dimension could vary little (Fig. 2c) or a great deal (Fig. 2d, f, h) with respect to the previous two dimensions. The third dimension could reveal that one member of the community is in fact different from the others (Fig. 2e), which was not readily apparent in only two dimensions (Fig. 2a). The third dimension could also be highly correlated with one of the previous two dimensions (Fig. 2g).

As depicted in Figure 3, use of the third dimension could prove essential in deciphering actual community structure and dynamics when new community members are introduced to a previously established community (Fig. 3e–h), as would happen with invasive species. The new species could vary from the original community in either the vertical dimension (Fig. 3c; in this example $\delta^{15}N$, which is indicative of trophic position) or in the horizontal dimension (Fig. 3d; in this example $\delta^{13}C$, which indicates organic carbon source), and this would then be reflected in the two dimensional metric values. However, if the new species did not differ from the previous community in either the vertical or horizontal dimensions (Fig. 3e), one could not infer changes to the community by these two dimensional metrics alone. The new species could be entirely similar to the previously established community (Fig. 3f), showing little change in metric values before and after invasion. In this case, even more dimensions would be necessary to determine if the new species alters the community structure. The new species could be similar to each other

and differ from the existing community in regards to a third dimension (Fig. 3g). The new species could not only differ from the existing community, but also vary in respect to each other in a third dimension (Fig. 3h).

The illustrations in Figure 2 and 3, demonstrate the need to analyze communities in more than two dimensions. Many distinguishing characteristics between communities are not readily visible when only two dimensions are used, in particular when trying to decipher feeding relations with isotopes, as the examples used here suggest. Although these demonstrations are in two and three dimensions, and the differences in community structure are easily distinguished using the plots alone, one could imagine a community in which you calculated the metrics with any number of dimensions (i.e. compound specific analyses) from which differences in community structure could not be gleaned from bi or tri-plots in an efficient manner.

For example, as detected in Figure 4, there were differences between the communities before and after dam construction not readily apparent from two dimensions. With the plethora of data obtained through compound specific analysis of amino acids, we had 24 dimensions from which to calculate metric values. Using Manhattan distances in the calculations of the metrics, we revealed how much new information was being gained by each new dimension (each new signature from an amino acid) through normalization and decomposition of the metrics. We learned that each novel dimension (amino acid signature) revealed a great deal of new information, some more than others as expected. This led to testing the relationships further. Using the $\delta^{13}C$ signatures from the amino acids and a Bayesian mixing model, FRUITS (Fernandes et al. 2014, 2015), we determined the proportion of different food sources utilized by the species in the Ohio River. Recalculating the metrics showed definite differences in the community before and after dam construction. For instance, before dam construction, the values

for SDNND and CHV were high, corresponding to a possible outlier with the other members of the community clumped together. After dam construction all metric values were lower, indicating that the species were more closely packed in isotopic niche space. Plotting three of the dimensions confirmed the species specific shifts and changes in overall community structure suggested by the metric values (Fig. 4).

These new community metrics also allow for the comparison of communities through time, as demonstrated with the Lower Ohio River fish samples (Fig. 4 & 5). Our example with real data not only further illustrates how the addition of another dimension can potentially show differences between two communities (Fig. 4) but also solves some of the shortcomings found (Hoeinghaus & Zeug 2008) with the metrics originally presented by Layman et al. (2007) (Fig. 5).

The isotopic ratios of nitrogen and carbon used in the original metrics of Layman et al. (2007) were meant to represent two different aspects of trophic structure, trophic position and the relative importance of basal source groups, respectively. Ideally each axis should have equal weighting when combined in metrics describing the overall trophic structure of a food web. However, distances along isotope axes represent different information. When ranges (NR and CR) are different or their variances are different, the other metric values (i.e. CD, NND, and SDNND) based on Euclidean distances will be more strongly affected by one of the two isotopes. This results in an artificial weighting of one aspect of trophic structure over the other (Hoeinghaus & Zeug 2008, Layman et al. 2012) Our community metrics, offering Manhattan distance calculations with the capabilities of normalization, solves many of the weighting issues previously afflicting the metrics of Layman et al. (2007).

Another limitation of the original metrics (Layman et al. 2007) is that the observed patterns could be a function of baseline variability and not reflect true differences among consumers, and metric values could be misleading or deceptive when basal source bulk signatures ($\delta^{13}C$) overlap. Rather than inferring food source use from relative spacing of consumers in $\delta^{13}C$-$\delta^{15}N$ bi-plots, an ecologist can now use baseline-corrected trophic position estimates instead of absolute $\delta^{15}N$ values in bivariate plots (with bulk-tissue signatures: Mercado-Silva et al. 2009, Swanson et al. 2010; with compound specific isotope analysis of amino acids: Chikaraishi et al. 2009, 2014, Bowes & Thorp 2015) and quantify the relative dietary importance of basal food sources using mixing models (e.g. FRUITS model, Fernandes et al. 2014, 2015), converting δ-space to %-space (dietary percent of basal sources; Fig. 5; Newsome et al. 2007). One can then use the our community metrics to quantify your community and compare between communities.

Moving beyond the qualitative descriptions of relative position in isotope-space (or trait-space, fatty-acid space, etc…), our community metrics provide a means for basic comparisons among food webs or other community properties. Although the demonstrations here are with isotope data and food webs, the analysis could easily be adopted for any number of analytical techniques. This simple extension and generalization of the metrics originally offered by Layman et al. (2007), with the advent of new technologies and the increase in availability of multidimensional data, can provide insight into community structure with little difficulty.

Our freely available, community metrics in R scripts are easily adapted to a number of different data types. The metrics allow for the comparison of communities, illuminating differences not readily apparent in one or two dimensions. These new metrics also allow for the comparison among communities through time or between different ecosystems, the only

requirements being each sample size (number of species) needs to be greater than the number of variables or axes, and that calculations of metric values be based on the same variables.

Figures and Figure Legends

Figure 1. The Manhattan distance function computes the distance that would be traveled to get from one data point to the other if a grid-like path is followed. The Manhattan distance between two items is the sum of the differences of their corresponding components.

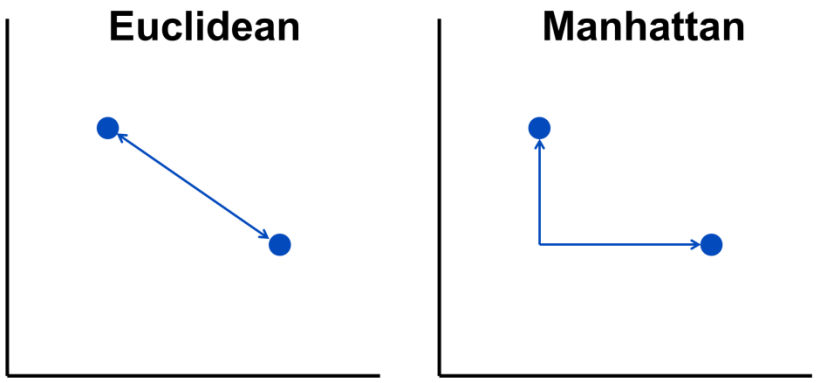

Figure 3-1 Manhattan vs. Euclidean distance.

Figure 2. Schematic stable isotope bi-plots ($\delta^{15}N$ and $\delta^{13}C$) of two communities in two (a–b) and three (c–h) dimensions. The first two dimension are shown in black, whereas the third dimension is represented by various colors corresponding to isotopic values ranging from extreme negative (-30) to barely negative (-5). Each point on the graph represents a species. Euclidean community metric values are included for comparison: CR, $\delta^{13}C$ range; NR, $\delta^{15}N$ range; IR, $\delta^{\#}I$ range; CD, mean distance to the centroid; NND, mean nearest neighbor distance; SDNND, standard deviation of nearest neighbor distance; CHV, total convex hull area (2 dimensions) or volume (more than 2 dimensions). a–b: The two theoretical communities look extremely similar in two dimensions (when only using $\delta^{15}N$ and $\delta^{13}C$), with each of the metric values confirming their similarity, all being nearly identical. c–h: When more information is added in the form of another

dimension (for example another isotope, $\delta^{\#}I$), it is evident that these two seemingly similar communities really differ from each other.

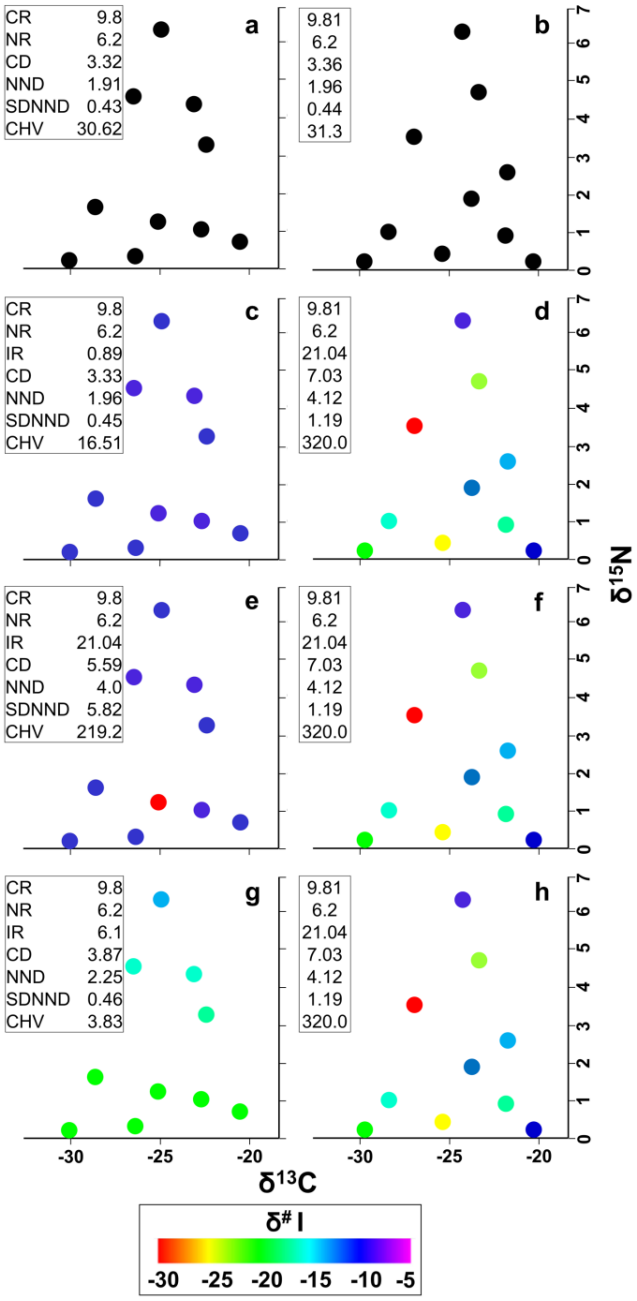

Figure 3-2 Schematic figure isotope bi- and tri-plots.

Figure 3. Schematic stable isotope bi-plots ($\delta^{15}N$ and $\delta^{13}C$) of conceptual ways three new species might affect the metric values of a community in two (a, c–e) and three (b, f–h) dimensions, with the third dimension being another possible isotope measured ($\delta^{\#}I$) and represented by colors corresponding to a range of values. Each point on the graph represents a species, with the existing community members as circles and the new species added as squares. Euclidean community metric values are included for comparison: CR, $\delta^{13}C$ range; NR, $\delta^{15}N$ range; IR, $\delta^{\#}I$ range; CD, mean distance to the centroid; NND, mean nearest neighbor distance; SDNND, standard deviation of nearest neighbor distance; CHV, total convex hull area (2 dimensions) or volume (more than 2 dimensions). a–b: The original community in two (a) and three (b) dimensions. In this example, all species are similar in respect to their third dimension ($\delta^{\#}I$) values, with IR being very small. c–e: Three examples of how three new species that are introduced into an existing community could alter community metrics in two dimensions. f–h: Three examples of how three new species introduced into an existing community that do not greatly influence metrics in two dimensions, could have a different or more significant impact when assessed by more dimensions (for example another isotope, $\delta^{\#}I$).

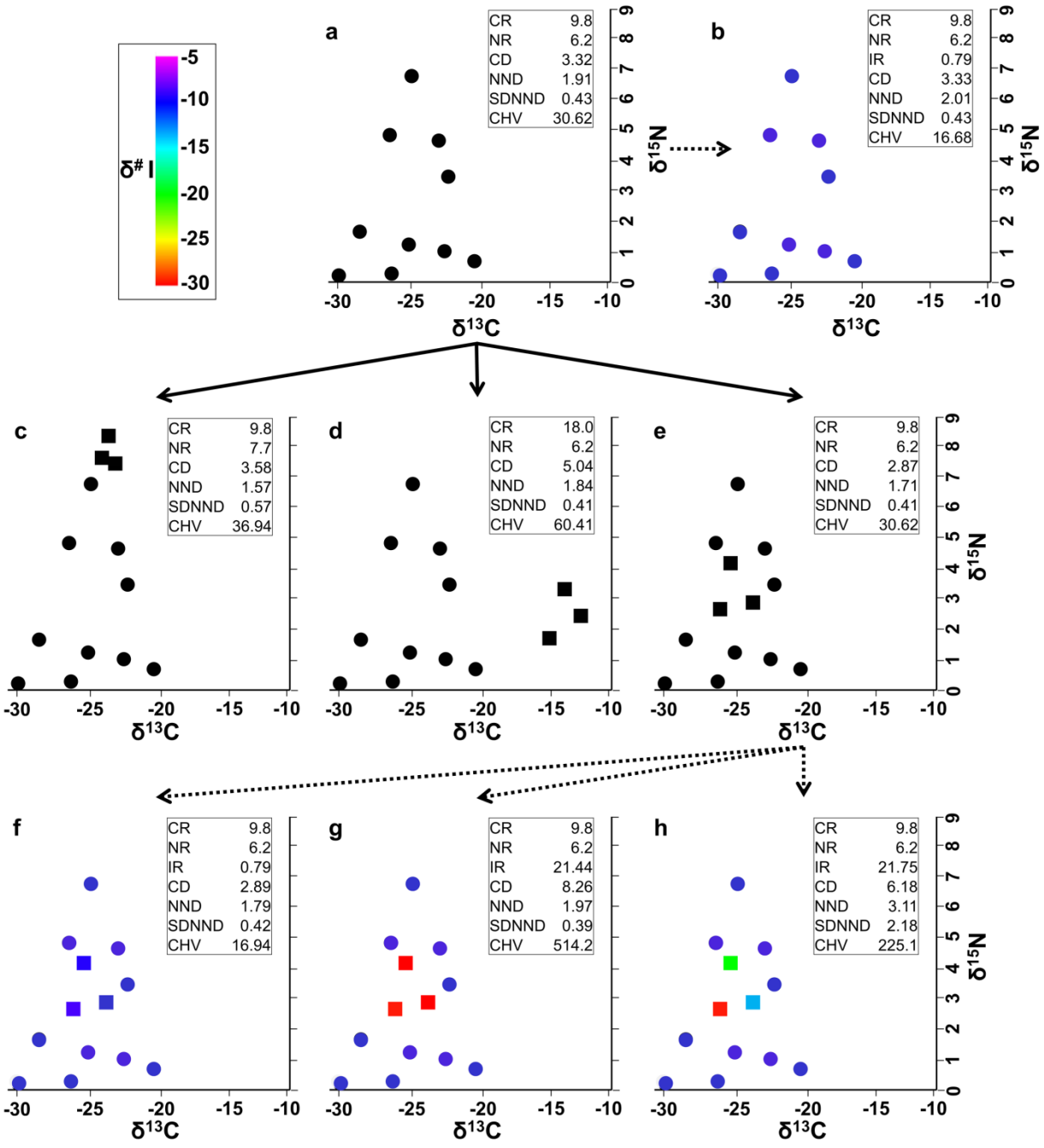

Figure 3-3 Schematic figure new community members.

Figure 4. a–b: Stable isotope bi-plots ($\delta^{15}N$ and $\delta^{13}C$) of a community of fish in the Lower Ohio River before and after major dam construction. Each point represents a species' mean value, with error bars removed for simplicity. Euclidean community metric values are included for comparison: CR, $\delta^{13}C$ range; NR, $\delta^{15}N$ range; IR, $\delta^{13}C$ range of an amino acid; CD, mean distance to the centroid; NND, mean nearest neighbor distance; SDNND, standard deviation of nearest neighbor distance; CHV, total convex hull area (2 dimensions) or volume (more than 2 dimensions). In two dimensions the community looks very similar before (a) and after (b) dams were built. c–d: Addition of a new dimension (i.e. based on a third chemical measure, in this case the $\delta^{13}C$ signature for the amino acid Glutamic Acid and represented by color corresponding to the range in isotopic values from -35 to -15) before (c) and after (d) dam construction. e–f: Addition of a new dimension (in this case the $\delta^{13}C$ signature for the amino acid Lysine, represented by color corresponding to the range in isotopic values from -35 to -15) before (e) and after (f) dam construction.

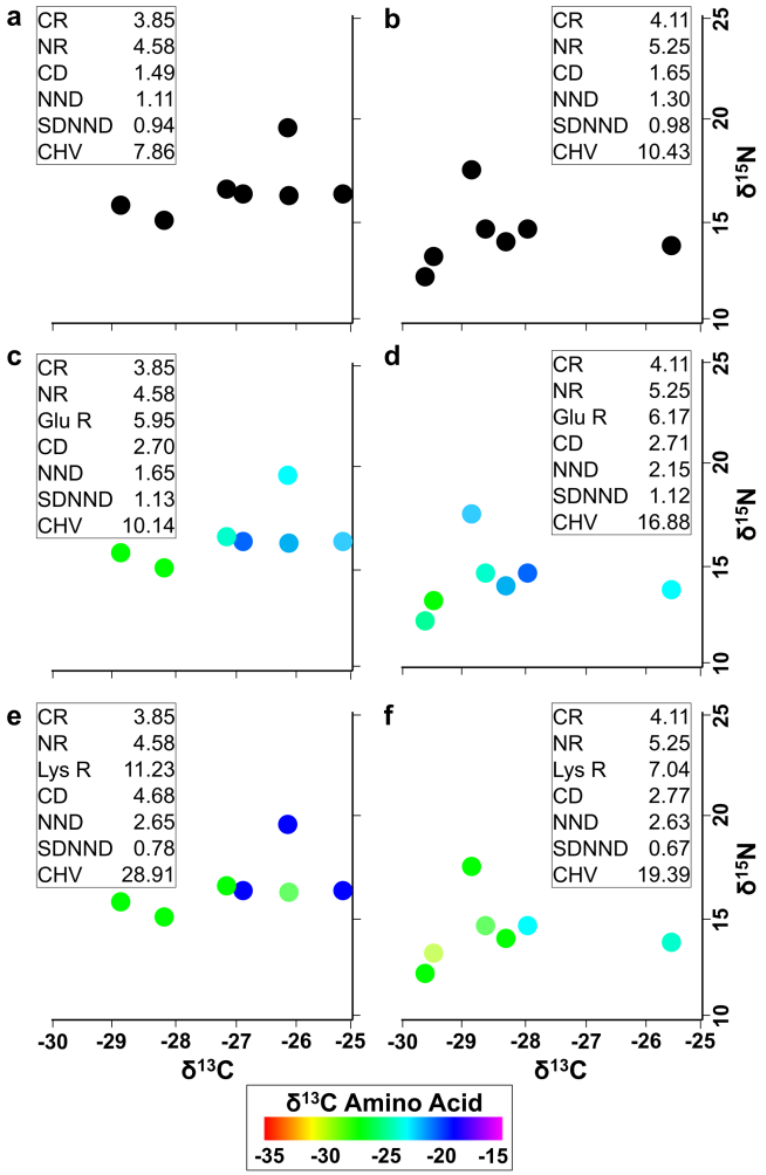

Figure 3-4 Lower Ohio River isotope bi- and tri-plots.

Figure 5. Fish in the Lower Ohio River before (a) and after (b) major dam construction. Plots consist of trophic position versus percent algae contribution to diet, evaluated by compound specific stable isotope analysis of amino acids ($\delta^{15}N$ and $\delta^{13}C$). Each point represents a species' mean value, with error bars removed for simplicity. Color shows percent C_3 terrestrial plant contribution to the diet. Euclidean community metric values are included for trophic position range (TPR); % algae range (AR); % C_3 terrestrial plants range (C3R);% C_4 terrestrial plants range (C4R); % aquatic macrophytes range (AMR); % cyanobacteria range (CR); mean distance to the centroid (CD); mean nearest neighbor distance (NND); standard deviation of nearest neighbor distance (SDNND); and convex hull volume (CHV).

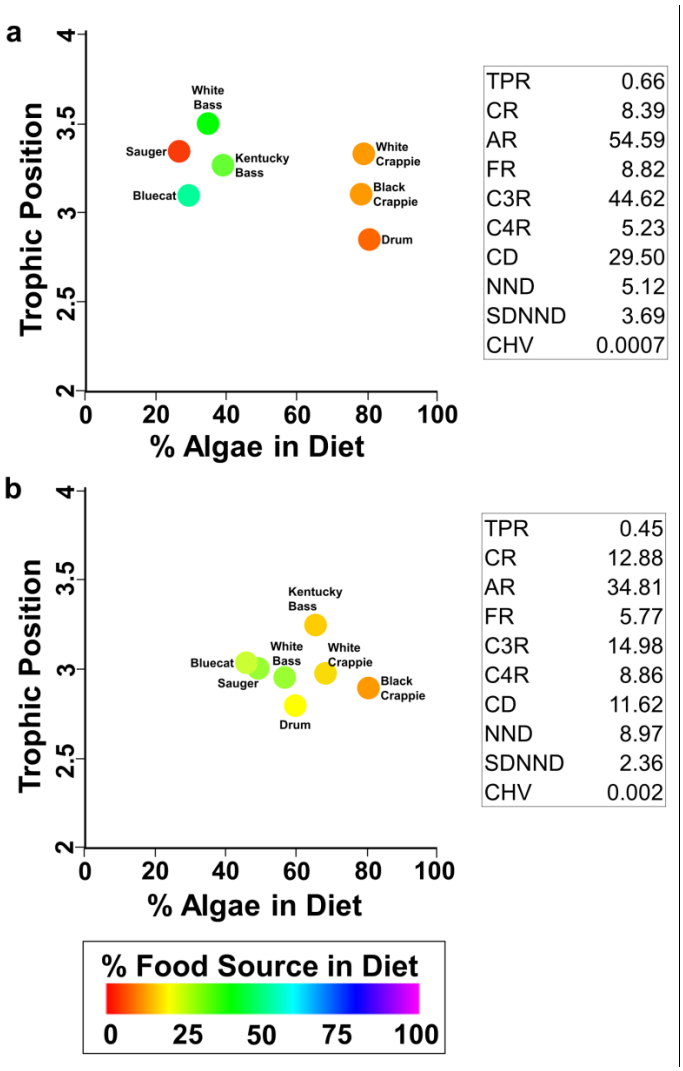

Figure 3-5 Lower Ohio River before and after dams.

Chapter 4

Epochal Changes to River Fish Trophic Positions

Abstract

Rivers are arguably the most abused ecosystems in North America as a result of damming, levee construction, species introductions, and pollution, but our historical knowledge of ecosystem change is limited when compared to research on lake and terrestrial ecosystems. The Upper Mississippi and Ohio rivers have some of the best long-term records in the USA and provide a unique opportunity to examine food web drivers over long time scales. We evaluated historical changes in trophic position of fish museum specimens using the newest and more precise technique of nitrogen amino acid stable isotope analysis. We found significant opposite shifts in trophic positions in the Ohio and Upper Mississippi rivers over a 50-yr period that were linked, by change-point analysis, with major alterations to habitat structure (e.g., abundance of side channels) resulting from construction of low-head dams. Discharge, gage height, and temperature were not correlated with the shift in trophic position. These two rivers naturally vary in hydrogeomorphic complexity (anastomosing vs constricted), and their discharge patterns differ both from each other (seasonal vs yearly operation in some cases) and from those characterizing high dams. It is not surprising, therefore, that factors controlling trophic position apparently vary between different types of dams and river structures.

Introduction

Rivers are among the most extensively altered ecosystems on earth. Over 60% of the world's large river basins are now affected by dams for irrigation, urban development, navigation, and energy production (Nilsson et al. 2005). Subsequent changes resulting from alteration of hydrological connectivity and natural flow regime have direct impacts to the biological, chemical, and physical properties of rivers and riparian environments.

The Upper Mississippi River (=UMR) and Ohio River (=Ohio) are part of the largest river system in North America, and both have been important for transportation, exploration, commerce, and water supply for centuries. Dams and locks in these rivers stabilize water levels during periods of low discharge, maintaining a minimum stage height (depth) needed for barge navigation. The magnitude and duration of high flows are relatively unchanged, however, because the dams are overtopped or all gates are opened during periods of high discharge (Chen & Simons 1986, Poff et al. 2007, Alexander et al. 2012).

Today's UMR and Ohio rivers are in many ways substantially different from those of just one hundred years ago (Figure 1). In the UMR, overall channel position and length have not changed substantially, but the number of islands has decreased due to erosion, especially in the lower portions of the navigation reach (Chen & Simons 1986, Delong 2005). Furthermore, the area immediately above the dams in the UMR consists mostly of open pools with simpler geometry and reduced dynamism because backwaters are lost due to water-level management, many sandbar complexes have disappeared, and there has been substantial loss of large woody debris (Grubaugh & Anderson 1988, Sparks 1995). Prior to construction of dams, the Mississippi River and its tributaries flooded low-lying lands of the floodplain adjacent to the river semi-annually and the entire valley bottom during extreme events (Yin & Nelson 1996, Delong 2005).

Above Clinton, Iowa 95% of the floodplain still connects to the UMR (Delong & Thorp 2006). Below Clinton, the construction of levees, lowering of the riverbed elevation, and, in some places, reduction of peak-discharge magnitudes have resulted in a nearly universal disconnection of the river main channel from the floodplain, except during extreme flood events (Pitlick 1997, Galat et al. 1998). In contrast, the channel complexity within our sampling areas of the Ohio has remained relatively constant over our sampling period, but the minimum and average water depths have increased. While the channel in this portion of the Ohio has remained somewhat constricted over time, a few more islands were present prior to the 1900s, especially at low water (Moody et al 2003, White et al. 2005).

Long-term studies are often vital for thoroughly assessing ecological impacts, but such research is limited by funding and the research tenure of the scientists who could study them. For example, reductions in lateral hydrologic connectivity have species-to-ecosystem level effects in rivers (Ward & Stanford 1995, Thorp et al. 2006, 2008), but documenting the effects can be challenging at any time, especially over periods greater than the academic career of most scientists, with most U.S. dams having been built in the 1960s and 1970s.

Food webs provide a means of analyzing both short- and long-term interrelationships among community structure, stability, and ecosystem processes, and how these attributes are influenced by environmental change and disturbance (DeAngelis 1992). Well-functioning food webs are fundamental in sustaining rivers as ecosystems and maintaining aquatic communities. Monitoring food webs and the variations occurring through time can tell us how organisms are influenced by changes to lateral hydrologic connectivity.

Although challenges to conducting food webs studies in extant communities of rivers are significant, they pale in comparison with understanding what has affected food webs over longer

time periods. Historical food webs studies are rare, notoriously difficult to undertake or interpret, and can be inconclusive or produce controversial results. However, recent studies (Delong et al. 2011, Turner et al. 2015) using traditional bulk-tissue isotope techniques have focused on long-term effects of high-head dams on food webs. In contrast, the historical effects of low-head dams on food webs have largely been ignored, even though these dams are much more common and have different impacts on lotic systems because of their size and operating procedures.

Hydrogeomorphic differences between high (> 15 m head) and low-head dams are well established, and we contend that these have substantially different effects on downstream food webs. High dams form an ecologically lake-like reservoir upstream from which water is intermittently released and sometimes pulsed diurnally for electric power production. Thermal characteristics of the released water can be substantially different, sometimes causing a shift in downstream species (e.g. Storey et al. 1991, Stevens et al. 1997). Moreover, the natural flow regime (Poff et al. 1997) is typically altered dramatically in terms of the amount and seasonal timing of peak flow (e.g., Delong et al. 2011). In contrast, the pools formed by low-head dams are much smaller in depth and surface area, water release is rarely pulsed, and effects on normal daily and seasonal flow patterns are minimal, especially in low-head navigation dams like those on the Ohio and Upper Mississippi (UMR) rivers. Consequently, the effects of these two general forms of river regulation on community composition and food webs should differ substantially.

We know from two previous stable isotope studies involving museum specimens collected over 70^{+} years (Delong et al. 2011, Turner et al. 2015) that high dams affect downstream food webs and that the putative causes are related to altered flow patterns and to some extent differences in nutrient releases. Turner et al. (2015) focused mostly on effects of river-floodplain engineering and nutrient addition in the Rio Grande system, finding evidence of

decreased isotopic niche breadth downstream from the dams. Delong et al. (2011) studied impoundment effects on various food web metrics immediately below the last high dam on the Missouri River. They noted that the seasonal amplitude of flow (reduced) and its seasonal periodicity (reversed annual high- and low-flow periods) exhibited the greatest change from the pre-dam to post-dam periods. They reported declines following dam construction in total community niche space, indicating a decrease in the range of basal resources and a shortening of the food chain. They concluded that the hydrologic, food web effects could have been due to: (a) reduced connectivity between main and side channels, thereby limiting access to potentially higher quality resources; and (b) altered food resource quantity and quality as a result of high flows during normally peak growing seasons.

The Upper Mississippi and Ohio rivers have some of the best historical samples in the USA and provided a unique opportunity to look at shifts in trophic position of piscivorous and invertivorous fish over long time scales. To gain a long-term perspective on resultant changes in trophic position (TP) and food chain length (FCL = maximum TP) from altering the physical environment of these major rivers with low-head dams, we used museum specimens of fish (e.g., Delong et al. 2011, Roussel et al. 2014). To improve the accuracy of our conclusions, we employed the newest techniques in compound specific, stable isotope analysis of amino acids (AA-CSIA) (e.g. Chikaraishi et al. 2007, 2009, 2014, Popp et al. 2007, Hannides et al. 2009, Steffan et al. 2013, Bowes & Thorp 2015). This technique obviates the need for independent $\delta^{15}N$ baseline information, which would be impossible to obtain for the historical range and sites analyzed. Furthermore, AA-CSIA has been found to be robust in aquatic food webs and more accurate than bulk isotope methods (Chikaraishi et al. 2009, Bowes & Thorp 2015).

Methods

Museum fish samples

Museum collections and species surveys by government agencies potentially provide data useful for analyzing long-term environmental impacts (Vander Zanden et al. 2003, Gido et al 2010), with the former being particularly valuable for studying effects on food webs (Delong et al. 2011). Although modern-day investigators are limited by historical variations in the species preserved, collection dates, and sites sampled, these restrictions can be ameliorated by careful development of the scope of study and the spatial and temporal scales over which specimens are selected (e.g., Vander Zanden et al. 2003).

For this study, we analyzed trophic state of piscivorous and invertivorous fish (Table 1) from > 300 km stretches of the UMR (Wabasha, Minnesota to Savanna, Illinois USA) and lower Ohio (Evansville, Indiana to Cairo, Illinois USA) using preserved specimens from museums located at the Bell Museum, Field Museum, Illinois Natural History Survey, Illinois State Museum, Milwaukee Public Museum, Ohio State University Museum of Biological Diversity, Southern Illinois University, University of Michigan Museum of Zoology, and University of Wisconsin – Stevens Point. Feeding tendencies of species were evaluated using information from state taxonomic keys for Missouri, Tennessee, and Wisconsin (Etnier 1993, Pflieger 1997, Becker 1983). The largest preserved specimens were chosen for tissue harvesting; however, museum specimens of fish tend to be smaller, as a consequence of the need to conserve valuable shelf space. Because of limits on the upper body size of some species, it is unlikely that some target species (especially piscivores) in the museum collections had fed exclusively within their identified feeding guild. Therefore, to evaluate food chain length changes over time, fish within the highest feeding guilds of piscivores and invertivores were chosen for analysis, as long as they were also consistently collected before and after dam construction. Tissue samples were initially

collected from individuals preserved from the late 1800s through early 2000s, but final samples selected for the present study were based on the consistency of species present over the periods 30-40 years before and after dam construction (1900s-1970s). These approaches minimized location-specific bias and interannual variability in the generation of food web measures.

Sample processing, isotope analysis, and trophic position calculation

Muscle tissue was extracted from between the lateral line and dorsal fin of adult fish species. Fishes were preserved in ethyl alcohol at the time of our sampling, but most were presumably preserved for short to long periods in formalin. However, the results of our analytical technique were not altered by this previous storage in formalin (Hannides et al. 2009, González-Bergonzoni et al. 2014). Tissue samples were rinsed with deionized water, placed in pre-combusted glass vials, dried at 60°C for 48 hr, and then ground into a fine, homogenized powder using a Wig-L-Bug® Mixer/Amalgamator.

After being dried, powdered, and homogenized, samples were analyzed for AA-isotope ratios at the UC-Davis Stable Isotope Facility. The general techniques for AA-isotope analysis are summarized below and described in greater detail in Walsh et al. (2014). Sample preparation involves acid hydrolysis for the liberation of amino acids from proteins and derivatization by methyl chloroformate to produce compounds amenable to GC analysis. Amino acid derivatives are injected in split (^{13}C) or splitless (^{15}N) mode and separated on an Agilent J&W factor FOUR VF-23ms column (30m X 0.25mm ID, 0.25 micron film thickness). Once separated, amino acid derivatives are quantitatively converted to CO_2 and NO_x in an oxidation reactor at 950°C, and NO_x are subsequently reduced to N_2 in a reduction reactor at 650°C. Following water removal through a nafion dryer, N_2 or CO_2 enters the IRMS. A pure reference gas (CO_2 or N_2) is used to

calculate provisional δ-values of each sample peak. Next, isotopic values are adjusted to an internal standard (e.g. norleucine) of known isotopic composition. Final δ-values are obtained after adjusting the provisional values for changes in linearity and instrumental drift such that correct δ-values for laboratory standards are obtained. The $\delta^{15}N$ of two amino acids (glutamic acid and phenylalanine) were determined by this method.

To calculate trophic position from the CSIA data, we employed the following formula: $TP = [((\delta^{15}N_{glutamic\ acid} - \delta^{15}N_{phenylalanine}) - 3.4) \div 7.6] + 1$ (e.g. Chikaraishi et al. 2007, 2009, 2014, Popp et al. 2007, Hannides et al. 2009, Steffan et al. 2013, Bowes & Thorp 2015).

Historical land use and water data

All historical water data for the Mississippi and Ohio rivers were obtained from the U.S. Geological Survey, 2014, National Water Information System, as accessed on August 29, 2014 from the World Wide Web (USGS Water Data for the Nation) at URL http://waterdata.usgs.gov/nwis/uv/?referred_module=sw. Historical land cover and use data were obtained from the U.S. Geological Survey, 2014, Land Cover/Use Data (USGS Data Visualization Tools, Upper Midwest Environmental Science Center), as accessed on November 11, 2014 from URL http://www.umesc.usgs.gov/data_library/land_cover_use/interactive_index.html.

Statistical analyses

Data were organized by the year the fish were initially collected from the field and preserved in the museum. After initial statistical analysis revealed a significant overlap in trophic positions of museum specimens of species traditionally identified as piscivores and invertivores

in both rivers (UMR: $F_{1,77} = 0.20$, $p = 0.658$; Ohio: $F_{1,60} = 3.67$, $p = 0.060$), we combined the two feeding guilds for final analyses. This allowed us to assess overall changes in trophic positions of fish from the highest two feeding guilds consistently collected over time in the rivers. Correlations between the value of TP for each fish specimen, as calculated by AA-CSIA, and the corresponding yearly average temperature, discharge, and gage-height data for both rivers were determined using Pearson correlation. Differences between TP before and after the major engineering project dates were tested using one-way ANOVA. All data were checked for normality, unusual values, and heterogeneity of variances. All statistics were performed in Minitab 14 statistical software (Minitab Inc., State College, PA, USA) with $\alpha = 0.05$.

We used change point analysis (Taylor 2000) to detect and characterize potential temporal differences in trophic position. The analysis uses a combination of cumulative sum charts and bootstrapping to detect changes, with the results including confidence levels and confidence intervals for changes in trophic position that may have occurred over time in the two rivers. A sudden, distinct change in the direction or sign of the slope in the cumulative sum plot indicates a sudden shift or change in the average (potential change points), which are then confirmed with bootstrapping.

Results

In the Ohio, the average trophic position of fish was significantly lower after dam construction ($F_{1,59} = 8.44$, $p = 0.005$; Figure 2a-b; Table 1). The change point analysis confirmed that a change occurred between 1947 and 1963, with 85% confidence (Figure 3). Although discharge slightly increased over time (Figure 2d), fish trophic positions were not correlated with mean annual discharge (m^3/s; $r = 0.069$, $p = 0.596$; Figure 2c), mean annual gage height (m; $r =$

0.222, p = 0.105; not shown), or mean annual air temperature (°C; r = 0.176, p = 0.175; not shown) during the period of record.

In the UMR, by contrast, we noted a significantly higher average trophic position of fish after dam construction of the 1930's ($F_{1,76}$ = 6.59, p = 0.012; Figure 4a-b; Table 1). The change point analysis also confirmed that a change occurred between 1927 and 1940, with 99% confidence (Figure 5). Fish trophic positions were not correlated with amount of discharge, and discharge did not vary significantly over time (m^3/s; r = -0.046, p = 0.692; Figure 4c-d). Similarly, we saw no correlation of trophic position with temperature or gage height (temperature: °C, r = 0.185, p = 0.105; gage height: m, r = -0.073, p = 0.578; both not shown).

Discussion

Historical Changes in Food Webs from Low-Head Dams

Given that low-head dams have minimal effects on the amount and timing of discharge and do not form true upstream reservoirs, does their presence modify riverine food webs or can other factors account for the changes we observed over the period of 30-40 years before and after dam construction (e.g., historical changes in temperature, nitrogen inputs, and river flow)? Our data indicated that trophic position was not correlated with variation in air/water temperatures over the period of study. Discharge patterns were not altered by the presence of these low-head dams, and long term patterns of TP/FCL showed no correlations with discharge fluctuations. The answer seems related to changes in the physical structure of a river which then affect food webs. As discussed below, however, this hypothesis is more complex than it may first appear because of differences between the UMR and Ohio in river form and both types of dams and their operation.

Puzzling Differences Between Food Webs of the Mississippi and Ohio Rivers?

The apparent effects of dams varied substantially between the UMR and Ohio. Trophic positions of invertivorous and piscivorous fish were lower after construction of the major hydrological engineering projects in the Ohio but higher in the UMR. Both rivers are regulated to maintain sufficient water depth for barge traffic during periods of low water flow. Because of this, both rivers maintain somewhat comparable, seasonal hydrological patterns, including responses to predictable flood pulses. Why, then, would low-head dam modification of the rivers elicit opposite shifts in fish trophic positions?

Our food web data suggest that the answer to this question does not involve any changes in mean annual gage height, temperature, or amount of discharge over time linked to construction of the dams on either river. Instead, the answer may partially lie in the fact that while both rivers are managed for navigation, the height and type of dams, their operation, and the hydrogeomorphic structure of the two rivers are different.

Navigation dams are higher on the Ohio, thereby creating a greater relative change in stage height/water levels than the dams on the UMR (White et al. 2005). The effect is that the larger, deep areas of slow-flowing water behind the dams on the lower Ohio reduce the relative amount of shallow areas in the photic zone, decreasing the effective area for benthic algal production while augmenting the volume for phytoplankton production. At the same time, channel complexity (mostly single-channel and somewhat constricted) does not vary substantially above and below dams on the Ohio even though water depths are greater immediately above the dams. The shift from a benthic to phytoplankton production in the Ohio has been documented with changes in carbon isotopes (Delong et al., unpublished manuscript)

Reductions in benthic algae would have impacted the secondary production and diversity of benthic invertebrates, and these changes could then be responsible for decreased trophic position of invertivorous and piscivorous fishes following dam construction.

The effects of dams on the UMR, by contrast, were substantially different. Dam and levee construction on the UMR changed the river from a system of meandering channels with some lateral complexity (islands, some secondary channels) to one with a relatively stable channel and extensive lateral complexity below each dam and downstream to a pool formed above the next dam downstream (Figure 1). This effectively increased the hydrogeomorphic complexity of the system, in-turn creating more diverse habitats for primary production and biota to thrive, leading to an increase in trophic positions of invertivorous and piscivorous fishes following dam construction (cf. Thorp et al. 2006, 2008).

Furthermore, the navigation pools in the UMR have aged, and these overwintering habitats, which were created when the pools filled, have declined as sedimentation continues to reduce water depth. This may account for the observed general trend of decreasing FCL and TP of fish of the UMR after original dam and levee construction.

Changes in the food webs in rivers can result from a variety of environmental factors, including altered hydrology and inputs from the surrounding watershed. For the present study, however, we contend that temporal changes in FCL and TP in these two large rivers resulted more directly from changes in habitat structural complexity than from any alterations in discharge patterns. We believe that management techniques that try to regulate the flow of the rivers to be more “natural” and pre-dam-like are worthy goals but are insufficient for restoring natural food web structure because TP seems to be more influenced in these cases by habitat complexity than discharge *per se.* Food chain length responds differently to different alterations

in hydrogeomorphic complexity and hydrologic connectivity, but all substantial alterations elicit a shift in FCL.

Figures and Figure Legends

Figure 1. Land cover and land use maps of the Mississippi River at Pool 8, located near La Crosse, Wisconsin (GIS data from USGS.gov). The 1891 map provides a look at the river prior to the development of the lock and dam system in the 1930's. The 1989 map displays the significant changes impoundment of the river introduced into the system.

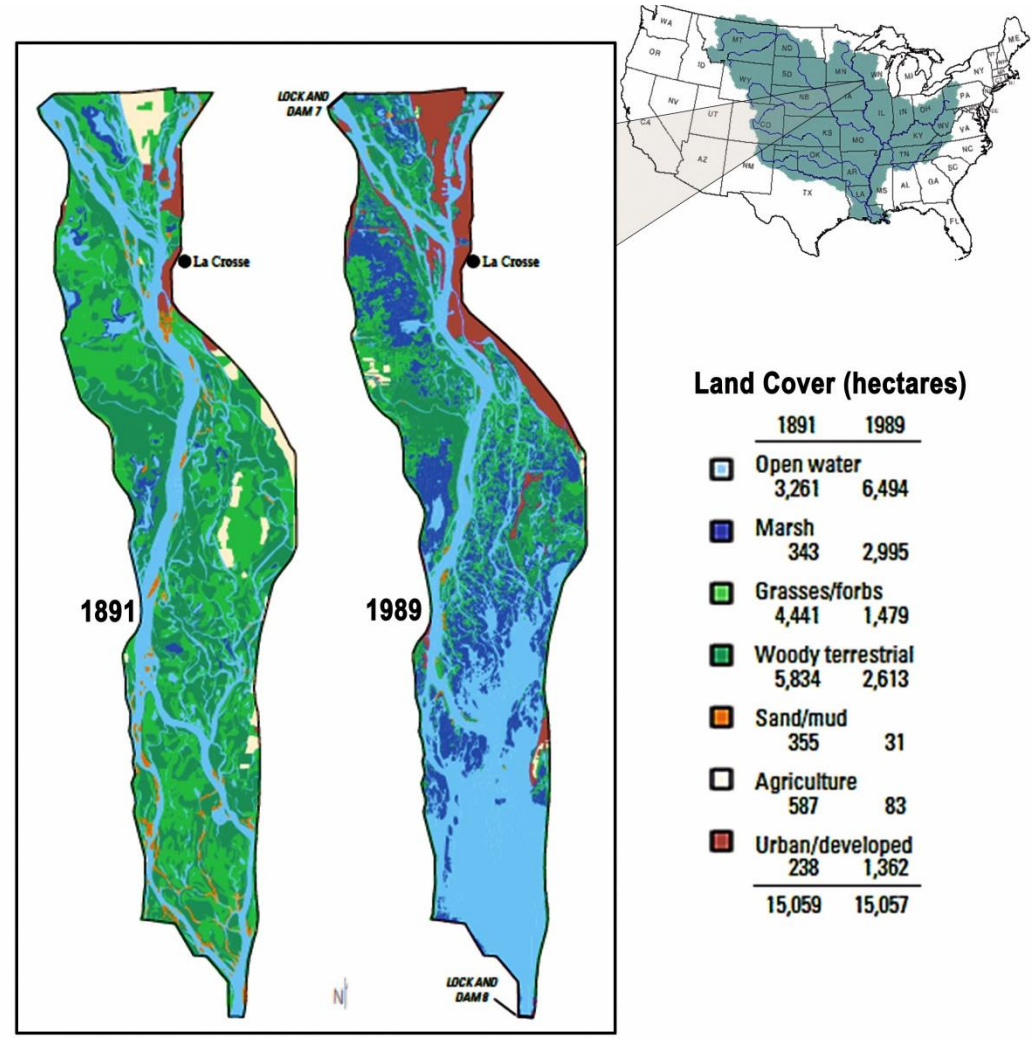

Figure 4-1 Map of land changes in UMR.

Figure 2. (A.) Trophic positions of invertivorous and piscivorous fish of the Lower Ohio River shifted following major lock and dam construction of the 1950's. (B.) Average trophic position of invertivorous and piscivorous fish of the Ohio significantly decreased from an average of 3.239 before (white background) to 2.980 after (gray background) the construction of the lock and dam system in the 1950's. (C.) Trophic position was statistically unrelated to corresponding average annual changes in discharge of the Lower Ohio River. (D.) Annual fluctuations and overall changes in discharge showed increasing trends over time, but was not the main driver of observed changes in trophic position of the fish.

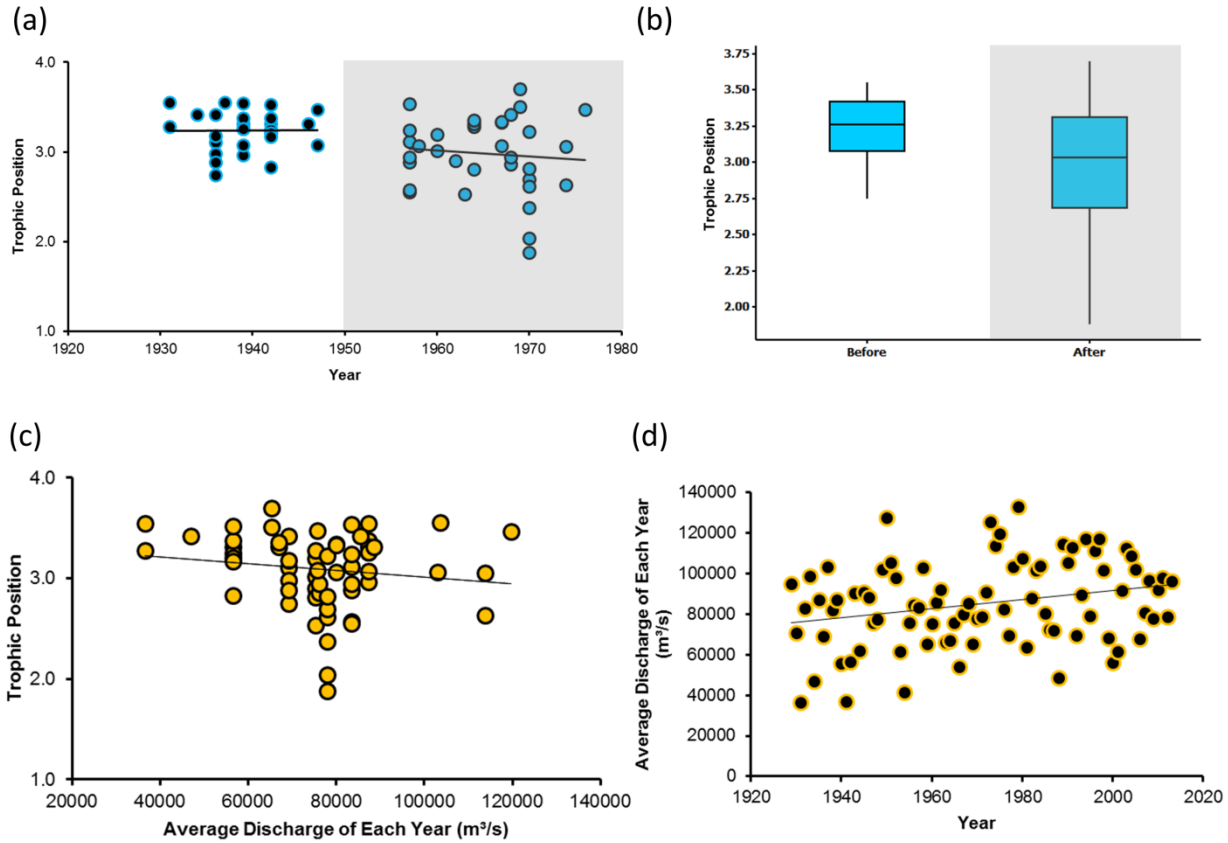

Figure 4-2 Trophic position changes in Ohio.

Figure 3. Graphical representation of the results of the change point analysis of fish trophic positions of the Ohio River. The shaded background represents the interval (1947-1963) between which a change occurred with 85% confidence.

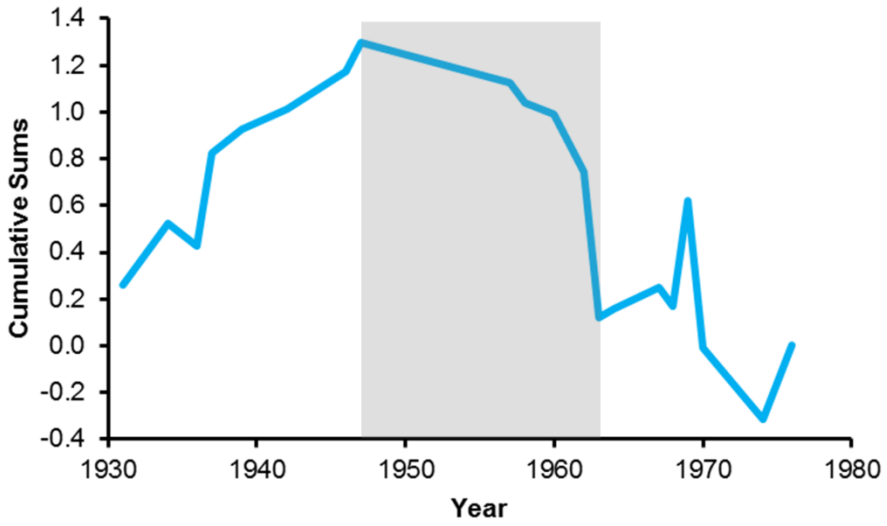

Figure 4-3 Change point analysis of Ohio.

Figure 4. (A.) A shift in the trophic positions of invertivorous and piscivorous fish of the Upper Mississippi River occurred in relation to major hydrological engineering construction of the 1930's. (B.) Average trophic position of invertivorous and piscivorous fish of the Upper Mississippi significantly increased from an average of 2.776 before (white background) to 3.037 after (gray background) the construction of the lock and dam system in the 1930's. (C.) Trophic position was unrelated to the corresponding average annual changes in discharge of the Upper Mississippi River, showing that single measures of hydrology may not capture the driver of change in river systems. (D.) Annual fluctuations and overall changes in discharge, temperature, and gage height showed increasing trends over time, but were not correlated with trophic position of the fish.

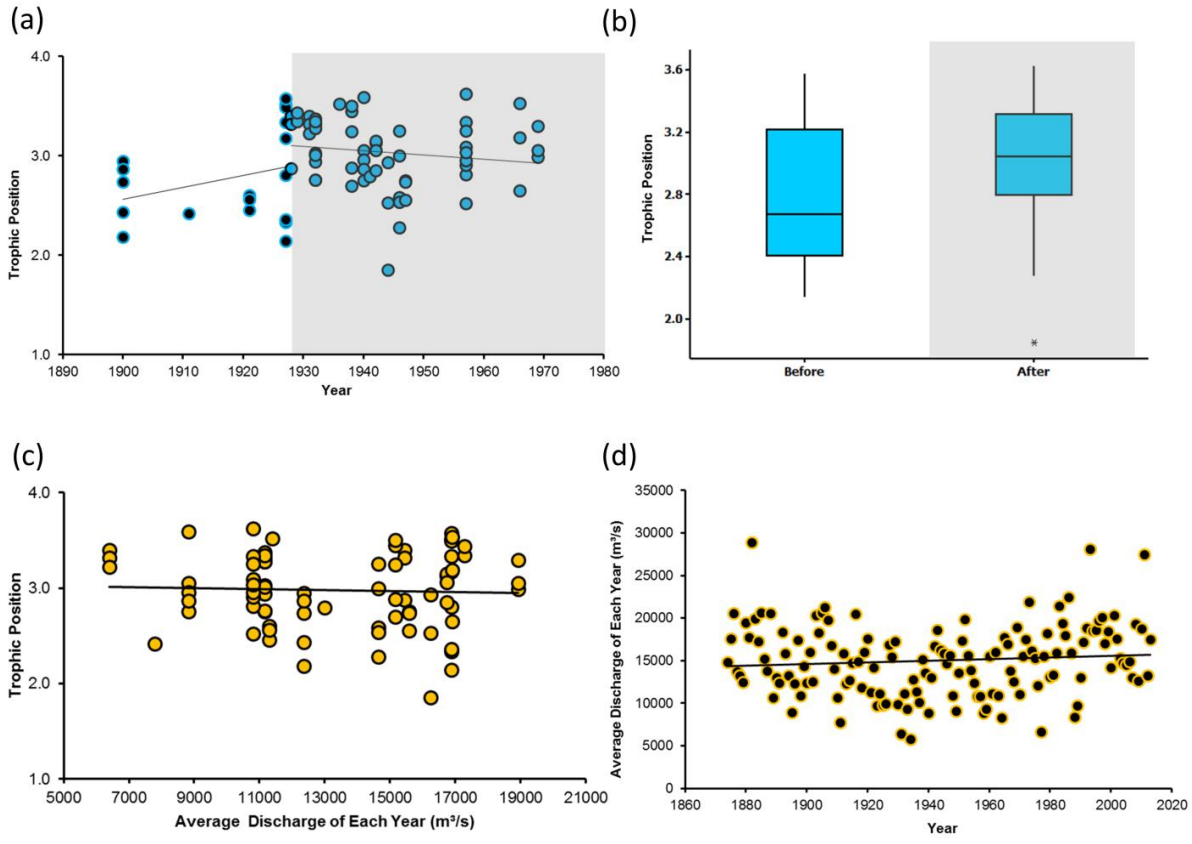

Figure 4-4 Trophic position changes in UMR.

Figure 5. Graphical representation of the results of the change point analysis of fish trophic positions of the Upper Mississippi River. The gray shaded background represents the interval (1927-1940) between which a significant change occurred with 99% confidence.

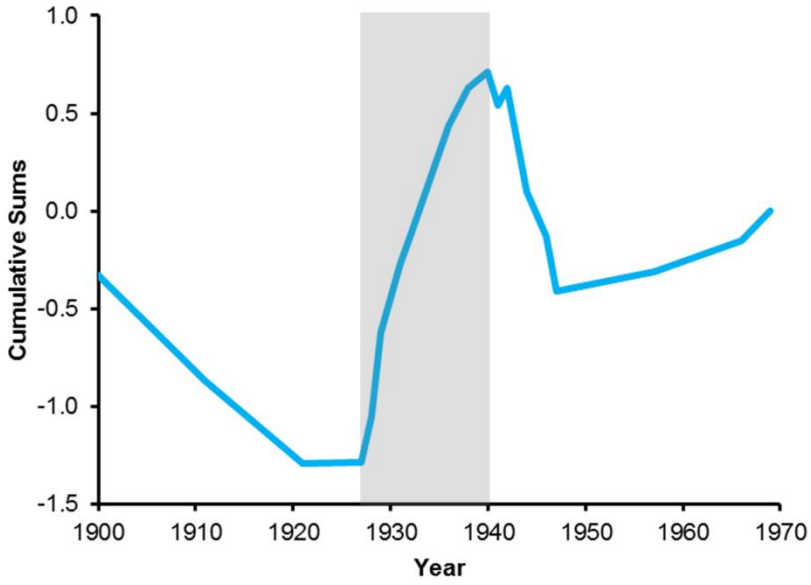

Figure 4-5 Change point analysis of UMR.

Tables

Table 1. Changes in mean trophic position of the invertivorous and piscivorous fish species collected from museums. Mean trophic position ± standard error (SE) of each species, as measured by $\delta^{15}N$ CSIA of amino acids, before and after lock and dam construction for each river (major dam construction occurred in the 1930's for the Upper Mississippi River (UMR), and in the 1950's for the Ohio River).

Fish Species	Before Dam Construction Mean ± SE Trophic Position	After Dam Construction Mean ± SE Trophic Position
Invertivores of UMR		
Pomoxis annularis	3.36 ± 0.07	3.23 ± 0.07
Pomoxis nigromaculatus	3.09 ± 0.24	3.16 ± 0.15
Aplodinotus grunniens	2.85 ± 0.09	2.45 ± 0.23
Percina shumardi	2.28 ± 0.08	2.89 ± 0.04
Piscivores of UMR		
Sander canadensis	3.05 ± 0.22	3.18 ± 0.16
Morone chrysops	2.20 ± 0.00	3.05 ± 0.14
Esox americanus	2.59 ± 0.07	2.72 ± 0.13
Sander vitreus	3.11 ± 0.20	3.60 ± 0.00
Invertivores of Ohio		
Pomoxis annularis	3.32 ± 0.10	2.97 ± 0.13
Pomoxis nigromaculatus	3.09 ± 0.12	2.89 ± 0.16
Aplodinotus grunniens	2.80 ± 0.00	2.79 ± 0.12
Ictalurus furcatus	3.08 ± 0.27	3.03 ± 0.16
Piscivores of Ohio		
Sander canadensis	3.33 ± 0.07	3.00 ± 0.35
Morone chrysops	3.49 ± 0.05	2.95 ± 0.54
Micropterus punctulatus	3.25 ± 0.09	3.24 ± 0.09

Table 4-1 Fish trophic positions of the Ohio and UMR.

Chapter 5

What fuels a river: carbon sources historically driving fish in the Mississippi and Ohio Rivers

Abstract

A 36^{+} yr debate on the primary origin of carbon supporting production and metabolism in lotic systems has spawned a number of conceptual theories, including the River Continuum Concept, the Flood Pulse Concept, the Riverine Productivity Model, the Riverine Ecosystem Synthesis, and the River Wave Concept. Testing of these theories has been hampered until now by the lack of adequate analytical methods to distinguish in consumer tissue between ultimate autochthonous and allochthonous carbon sources. Now, however, the newest technique in amino acid compound specific isotope analysis (AA-CSIA) directly and reliably enables investigators to link consumers to ultimate food sources by tracing essential amino acids, compounds that cannot be created by animals and must be ingested. We tested predictions of these five conceptual theories by using AA-CSIA to analyze carbon sources in tissue of 5 invertivorous and 6 piscivorous species from museum samples collected over decades from the anastomosing Upper Mississippi River and the mostly constricted lower Ohio River.

Our results demonstrate that ~80% of 4 essential and 5 non-essential amino acids were ultimately derived from autochthonous algae in these two hydrogeomorphically disparate large rivers rather than from allochthonous, terrestrial carbon. Moreover, results from the 11 species examined individually consistently demonstrated the dominant importance of algae for mid-to-upper trophic guilds, even though some significant differences existed in carbon use among species and rivers. Our results are consistent with general conclusions on the importance of autotrophy reported for a number of rivers around the world based on bulk tissue isotope and fatty acid methods, but our current data for the first time reveal the surprisingly high and consistent degree to which algal sources contribute to food webs in two hydrogeomorphically distinct rivers.

Introduction

The Upper Mississippi River (=UMR) and Ohio River (=Ohio) are part of the largest river system in North America, and have changed drastically over the past century. This is not surprising, considering nearly all large river systems in the world are now impacted to some degree by anthropogenic activities (Jackson et al. 2001, Nilsson et al. 2005). Channel morphology and sediment transport dynamics have been altered by years of flow regulation by dams, levees, and reservoirs (Ligon et al. 1995, Ward & Stanford 1995a, Lytle & Poff 2004, Kondolf & Podolak 2014). Agriculture and urbanization have led to massive nutrient additions and pollution. This, along with channelization usually results in spatial and temporal simplification and homogenization of floodplain river ecosystems (Rahel 2000, Moyle & Mount 2007). Homogenization changes productivity and the distribution of resources available to consumers, diminishes river channel complexity, and decreases the potential for interaction with riparian floodplain habitats, thereby changing nutrient and energy flow throughout the entire river ecosystem (Power et al. 1995, Ward & Stanford 1995b, Finlay 2001).

Documenting long-term changes in aquatic ecosystems is notoriously difficult. Food webs, being a central organizing theme in ecology, are special descriptions of biological communities focused on trophic interactions between consumers and resources. They provide a means of analyzing interrelationships among community structure, stability, and ecosystem processes, and how these attributes are influenced by environmental change and disturbance (DeAngelis 1992). Well-functioning food webs are fundamental in sustaining rivers as ecosystems and maintaining associated aquatic and terrestrial communities.

Food webs in streams and rivers are often complex, incorporating aspects of both terrestrial and aquatic systems. River food sources, such as algae and terrestrial matter, are

heavily influenced by seasonal changes, flow conditions and anthropogenic activity (Dodds & Whiles, 2010), resulting in spatial and temporal variation in food availability. This leads to a high degree of omnivory and mixed feeding modes for freshwater consumers, particularly for those in running waters (Woodward et al. 2005, 2010, Anderson & Cabana 2007). This flexibility makes interpreting consumer–resource relationships in rivers complicated.

Understanding the relative importance of allochthonous (terrestrial) and autochthonous (aquatic) sources of carbon that support the food webs of large rivers is essential for nearly every aspect of river engineering and floodplain management (Park et al. 2003, Dudgeon 2010, Wang et al. 2014). There are several conceptual models have been proposed to examine contributions of various carbon sources to river food webs. The river continuum concept proposes that the major source of organic matter supporting large river food webs originates from terrestrial plants, while in-stream primary production is limited by light reduction associated with depth and turbidity (Vannote et al. 1980, Sedell et al. 1989). About 80% of all primary production eventually enters the detritus food web (Pomeroy 1991) and exceeds algae-based production (Gushing et al. 2006). Some evidence exists to support the notion that terrestrially derived organic matter is an essential source of carbon and may even dominate aquatic consumers in streams (Wallace et al. 1997, Reid et al. 2008) and rivers (Huryn et al. 2001, Hoffman et al. 2008, Zeug & Winemiller 2008, Wang et al. 2014). The flood pulse concept highlights the importance of lateral river floodplain exchanges and proposes that river food webs are more dependent on production derived from the floodplain than on organic matter transported from upstream (Junk et al. 1989). The riverine productivity model (Thorp & Delong 1994) emphasizes the importance of local in-stream production (phytoplankton, benthic algae, and other aquatic plants). Several studies have identified algal carbon as an important carbon source fueling river

food webs around the world (Hamilton et al. 1992, Sobczak et al. 2002, Bunn et al. 2003, Thorp & Delong 2002, Brett et al. 2009, Pingram et al. 2014). Furthermore, results of a study on the Ohio River showed that the riverine productivity model better explained energy sources within a floodplain and constricted-channel reaches (Thorp et al. 1998). Considering the existence of opposing findings on the relative contribution of terrestrial and aquatic carbon sources to river food webs, additional work is required to gain insight into the energy flow and nutrient cycling pathways of large rivers, and how reliance on particular basal carbon sources is affected by river engineering.

Attempts to test these food web models have primarily involved stable isotope analysis, lipid (or fatty acid) analysis, or gut content analysis. The first two approaches measure the amount of nutrients assimilated into the consumer over long periods (weeks to years) but typically do not reveal either the exact prey or what was eaten recently (e.g., Ruess et al. 2005, Iverson et al. 2009, Richoux et al. 2014). In contrast, an analysis of gut contents reveals diurnal feeding success and may identify the exact prey item; however, it does not indicate which organic items in the gut are actually assimilated and does not necessarily reflect average diet over longer periods (Davis & Munoz 2016). To gain a long-term perspective on changes in food webs, one option is to use museum specimens of fish (e.g., Vander Zanden et al. 2003, Delong et al. 2011) and stable isotope analysis.

The Mississippi and Ohio rivers have some of the best historical samples in the USA, and thus provide a unique opportunity to look at changes in food sources over long time scales. Here we look at the potential of compound specific stable isotope analysis of amino acids (AA-CSIA) of $\delta^{13}C$ in determining basal food sources in river food webs. Then we use this isotopic

fingerprinting method in conjunction with a Bayesian mixing model to track changes in food source use in the Ohio and UMR through historical time.

Methods

Museum fish samples

Museum collections and species surveys by government agencies provide data potentially useful for analyzing long-term environmental impacts (Vander Zanden et al. 2003, Gido et al. 2010) as well as spatially dispersed ecological processes. Although present-day investigators are limited by historic variations in the species preserved, collection dates, and sites sampled, these restrictions can be ameliorated by careful development of the study scope and the spatial and temporal scales over which specimens are selected (e.g., Vander Zanden et al. 2003).

We analyzed food sources and trophic state of piscivorous and invertivorous fishes from greater than 300 km stretches of the UMR (Wabasha, Minnesota to Savanna, Illinois USA) and Ohio (Evansville, Indiana to Cairo, Illinois USA) using preserved specimens from museums. Samples were donated by the Bell Museum, Field Museum, Illinois Natural History Survey, Illinois State Museum, Milwaukee Public Museum, Ohio State University Museum of Biological Diversity, Southern Illinois University, University of Michigan Museum of Zoology, and University of Wisconsin - Stevens Point. To establish initial diet tendencies of species we consulted state taxonomic keys for Missouri, Tennessee, and Wisconsin (Etnier 1993, Pflieger 1997, Becker 1983). In total, we analyzed food sources for 4 species (29 individuals) of invertivores and 5 species (37 individuals) of piscivores originally collected (1900-1969) from the UMR, and 4 species (30 individuals) of invertivores and 4 species (24 individuals) of piscivores originally sampled (1931-1970) from the Ohio.

Museum-archived field collections are usually made without a priori statistical or experimental design, and we expected additional variance to be introduced into the study as a result. To avoid this, only the largest preserved specimens were chosen for tissue harvesting; however, museum specimens of fish tend to be small in general, reflecting the need to conserve limited shelf space. Because of body size restrictions, it is likely that some of these piscivores were more omnivorous (i.e., feeding also on invertebrates) than their guild placement would suggest. This was also confirmed by the calculated trophic positions of insectivorous and piscivorous fish overlapping in both rivers. Based on AA-CSIA data (methods described below), the piscivores in general had only a slightly higher trophic position than the invertivores in both the UMR (TP: 3.00 vs 2.95; $F_{1,76} = 0.24$, $p = 0.628$) and the Ohio (TP: 3.20 vs 3.01; $F_{1,58} = 3.67$, $p = 0.060$). Therefore, we combined these groups in analyses.

Sample processing and isotope analysis of fish tissue

We extracted muscle tissues from an area between the lateral line and dorsal fin of adult fish preserved in today's museums in ethyl alcohol and probably previously for short or long periods in formalin. Neither preservative significantly alters the AA-CSIA results (Hannides et al. 2009, González-Bergonzoni et al. 2014). Tissue samples were rinsed with deionized water, placed in pre-combusted glass vials, dried at 60°C for 48 hr, and then ground into a fine, homogenized powder using a Wig-L-Bug® Mixer/Amalgamator.

After samples were dried, powdered, and homogenized, their amino acid stable isotope ratios were determined at the UC-Davis Stable Isotope Facility. General techniques for AA-CSIA are summarized below and extensively described in Walsh et al. (2014). Sample preparation involves acid hydrolysis for the liberation of amino acids from proteins and

derivatization by methyl chloroformate to produce compounds amenable to GC analysis. Amino acid derivatives are injected in split (^{13}C) or splitless (^{15}N) mode and separated on an Agilent J&W factor FOUR VF-23ms column (30m X 0.25mm ID, 0.25 micron film thickness). Once separated, amino acid derivatives are quantitatively converted to CO_2 and NO_x in an oxidation reactor at 950°C, and NO_x are subsequently reduced to N_2 in a reduction reactor at 650°C. Following water removal through a nafion dryer, N_2 or CO_2 enters the IRMS. A pure reference gas (CO_2 or N_2) is used to calculate provisional δ-values of each sample peak. Next, isotopic values are adjusted to an internal standard (e.g. norleucine) of known isotopic composition. Final δ-values are obtained after adjusting the provisional values for changes in linearity and instrumental drift such that correct δ-values for laboratory standards are obtained. Signatures of both $\delta^{13}C$ and $\delta^{15}N$ were determined for the following amino acids and expressed as per mil (‰): Alanine, Aspartic Acid, Glutamic Acid, Glycine, Isoleucine, Lysine, Methionine, Phenylalanine, Proline, Tyrosine, and Valine. Tyrosine, Methionine, and Lysine signatures were excluded from analyses dues to missing measurements caused by concentrations below detection limits in the food sources measured, although they were present in the fish samples.

To calculate trophic position of consumers from AA-CSIA data, we employed the following formula: TP = [((^{15}N of glutamic acid - ^{15}N of phenylalanine) - 3.4) ÷ 7.6] + 1 (e.g. Chikaraishi et al. 2007, 2009, 2014, Popp et al. 2007, Hannides et al. 2009, Steffan et al. 2013, Bowes & Thorp 2015).

Food source calculations

To calculate the amino acid composition of food sources, we measured isotopic signatures using $\delta^{13}C$ AA-CSIA for three replicates of the following potential aquatic and

terrestrial food sources, as represented biochemically by: cyanobacteria (*Spirulina*), green algae (*Chlorella sp.*), fungi (baker's yeast or *Saccharomyces cerevisiae*), C_3 terrestrial plants (C_3 grasses (*Elymus sp.*, probably *E. virginicus*), C_3 tree leaves (cottonwood, *Populus deltoides*), two C_3 crops (soybean, *Glycine max*)), a C_3 aquatic vascular macrophyte (wild celery, *Valisneria americana*), and a C_4 terrestrial plant (corn, *Zea mays*). These specific food sources were chosen as they represent common food sources available in rivers across the USA. The terrestrial sources were collected in Lawrence, Kansas, and aquatic sources were ordered from laboratory cultures (PureBulk.com). These new signatures were used in conjunction with data from other aquatic studies (Larsen et al. 2009, Larsen et al. 2013) to determine classification and specific isotopic fingerprints of the different food sources (Fig. 1, Fig. 2).

$\delta^{13}C$ values of each of the amino acid were normalized to their respective sample means ($\delta^{13}C_{AA}$ – mean $\delta^{13}C_{AA}$), and tested for univariate normality. Normalizing the values to the means removes any effect of growth media between the different food sources. To explore patterns and determine producer food groups, we performed a cluster analysis and principle component analysis on normalized $\delta^{13}C$ signatures of all available amino acids, this showed that samples clustered according to major phylogenetic associations (5 major groups were identified: cyanobacteria, algae, fungi, C_3 terrestrial and C_3 aquatic macrophyte, and C_4 terrestrial; Fig. 2). Differences in each amino acid $\delta^{13}C$ signatures between these different producer groups were tested with ANOVA. We then performed linear discriminant function analysis on $\delta^{13}C$ AA-CSIA to determine the combination of $\delta^{13}C$ AA-CSIA values (independent variables, in this case 9 amino acids: Alanine, Aspartic Acid, Glutamic Acid, Glycine, Isoleucine, Lysine, Phenylalanine, Proline, and Valine) that best explained differences between food sources (categorical variables determined by principle component analysis), and we used a leave-one-out cross validation

approach to calculate the probability of food source group membership of the classifier samples. To test that there were no difference in classification between the groups Pillai-Bartlett trace (MANOVA) was applied. All preliminary analyses on food sources were done in Minitab 14 (Minitab Inc., State College, PA, USA).

Relative contributions of dietary amino acids to consumers were estimated using the software "Food Reconstruction Using Isotopic Transferred Signals" (or FRUITS; Fernandes et al. 2014, 2015). Normalized $\delta^{13}C$ values as well as their associated uncertainties (±1 S.D.), for each consumer species and potential food sources (absolute values found in Table 1) in the river were input into the FRUITS model. FRUITS can account for dietary routing; that is, the contribution of different original primary production sources towards the amino acids signals measured in the consumer. It was assumed that all food sources were equally likely and had the potential to make up 100% of the diet of the consumer. Based on values reported in Larsen et al. (2013), biochemical compositions of amino acids were taken into account. No other priors were used in the model. FRUITS is executed with a software package for performing "Bayesian inference Using Gibbs Sampling" (or BUGS), and also considers the biochemical composition of sources and which sources are most likely to contribute the most (see http://www.mrc-bsu.cam.ac.uk/software/bugs/). The FRUITS output is a summary of percent contributions of each potential food source to the consumer's diet along with standard deviation and confidence intervals. FRUITS version 2.0 (http://sourceforge.net/projects/fruits/) was used for estimating food source contributions. Sensitivity analyses were conducted to evaluate the reliability of the results by taking into account posterior uncertainties in the proportional contributions of different food sources and food source combinations (Fernandez et al. 2014).

Results

Food Source Classifications

Major food source groups were identified as: cyanobacteria, algae, fungi, C_3 terrestrial, C_4 terrestrial, and aquatic macrophyte, with each exhibiting very different patterns of $\delta^{13}C$ variation among both essential and nonessential amino acids (Fig. 1). For both essential and nonessential amino acids, taxon identity, amino acids identity, and their interaction had highly significant effects on amino acid $\delta^{13}C$ (all $p<0.001$). The presence of a highly significant interaction between taxon and amino acid demonstrates that isotopic variations among individual amino acids were taxon dependent. Linear discriminant analysis revealed highly significant differences between taxa, based on non-normalized $\delta^{13}C$ values from 4 essential (Pillai trace = 2.18455, $F_{4.23} = 5.716$, $p = 0.000$) and 5 non-essential (Pillai trace = 3.03819, $F_{4.23} = 11.372$, $p = 0.000$) amino acids. Five food sources had distinct isotopic clusters for each taxa when graphed with the first (accounting for 80.1% of the variation) and second (accounting for 14.3% of the variation) discriminant axes (Fig. 2). All food groups classified with >99.99% certainty and posterior probability with their own groups (Fig. 2).

Ohio River Food Source Changes

There was a lot of variability in food source use of the fish of the Ohio River through time (Fig. 3). Though, overall food source use before and after major hydrological construction projects was similar in the Ohio River (Figure 4; before dam: Algae = 73.63% ± 4.88% (SD), Cyanobacteria = 1.247% ± 1.244%, Fungi = 3.09% ± 2.521%, C_3 terrestrial = 7.18% ± 5.759%, C_4 terrestrial = 9.781% ± 3.461%; after dam: Algae = 77.72% ± 7.49% (SD), Cyanobacteria = 1.248% ± 1.314%, Fungi = 6.155% ± 3.509%, C_3 terrestrial = 4.396% ± 4.756%, C_4 terrestrial =

6.928% ± 3.744%). Species specific shifts in diet and trophic position were evident in the LOR before and after dam construction (Fig. 5).

Upper Mississippi River Food Source Changes

Before dam construction on the UMR, there was very little variability in food source use of the fish, as compared to the high variability afterward (Fig. 6). However, overall food source use before and after major hydrological construction projects was similar in the UMR (Figure 7; before dam: Algae = 88.15% ± 2.881% (SD), Cyanobacteria = 1.434% ± 1.264%, Fungi = 2.545% ± 1.649%, C_3 terrestrial = 2.82% ± 2.568%, C_4 terrestrial = 2.651% ± 1.704%; after dam: Algae = 84.94% ± 1.815% (SD), Cyanobacteria = 1.01% ± 0.9406%, Fungi = 7.398% ± 2.924%, C_3 terrestrial = 1.533% ± 1.417%, C_4 terrestrial = 3.634% ± 2.839%). Species specific shifts in diet and trophic position were also evident in the UMR before and after dam construction (Fig. 8).

Discussion

FRUITS and CSIA-AA to determine basal carbon sources in rivers

Our results show that $\delta^{13}C_{AA}$ patterns can be used as a powerful tool in tracing food sources through river food webs (Fig. 1, Fig. 2). We found that $\delta^{13}C$ values for nine amino acids were significantly different between five food types. While the exact mechanism explaining why these different food types (C_3 plants, terrestrial C_4, aquatic algae, bacteria, and fungi) have such distinct $\delta^{13}C_{AA}$ patterns is not currently clear (Larsen et al. 2013), it does provide a strong diagnostic potential in distinguishing ultimate carbon sources in organisms higher in food webs.

Utility of museum specimens in river food web studies

Museum specimens are a powerful tool in addressing questions of community and ecosystem change over decadal time scales (e.g., Vander Zanden et al. 2003, Schmidt et al. 2011, Turner et al. 2015). Museum collections can provide a baseline to estimate changes over time in large rivers, where reference systems for comparative studies are nonexistent, as well as potentially be useful in parameterizing models of river ecosystem function (Power et al. 1995b). Efforts are ongoing in the ecological and natural history communities to archive specimens from long-term monitoring studies, which are likely to provide powerful future insight into global and regional climate change, alteration of landscapes, and other natural and anthropogenic mediated drivers of community and ecosystem function (Turner et al. 2015).

Autotrophy is the dominant source of carbon for food webs in the UMR and Ohio

Our data show that allochthonous material, while important for certain consumer groups, is not the basis of fish production in the studied ecosystems. Results of our study demonstrate that instream autotrophic organisms are the originating and overwhelmingly dominant, biotic source of carbon for the 11 species of piscivorous and invertivorous fish collected from these two large rivers, the constricted lower Ohio River (Ohio) and the anastomosing Upper Mississippi River (UMR). Given that hydrogeomorphic structure of rivers is thought to extensively influence ecological processes (Poole 2002, 2010, Thorp et al. 2006, 2008), we expected the use of algae to be high in both rivers but greater in the UMR. The degree of importance of algae to secondary production will undoubtedly vary somewhat among rivers of different hydrogeomorphic structures, but we predict that autochthonous carbon will provide the primary support of secondary production in most large rivers, with the possible exception of

rivers where instream autotrophy is substantially suppressed by light limitations (high turbidity, great depth, or riparian cover). Seasonal differences will alter algal contributions somewhat; but on an annual basis, autochthony still dominates. It is possibly still surprising that large river food webs are often dependent on the low biomass of algae, but as the research emphasis has changed from consumer ingestion to assimilation, it has become increasingly clear that food quality, and not quantity, is the more important factor regulating the efficiency of energy flow through river ecosystems (Torres-Ruiz et al. 2007, Lau, Leung & Dudgeon 2008, 2009, Brett et al. 2009, Lau et al. 2009). Allochthonous food sources, such as leaf litter, and fungi and bacteria colonizing leaves, are considered lower quality food for invertebrates, on which many of the fish in study likely fed on, compared with algae because of algae's lower C : N and C : P ratios (Frost & Elser 2002, Cross et al. 2005, Torres-Ruiz et al. 2007, Lau et al. 2009).

Historical changes in food sources in the Ohio

Our data suggest that although the Ohio River has changes in structure, with the addition of lowhead dams, and hydrology, with regulation of discharge and stage height, the basal food sources used by invertivorous and piscivorous fish have changed minimally. The Ohio has a high variability in resource use by consumers from year to year, and species shift in their basal carbon source through time (Fig. 3, Fig. 5). Navigation dams are higher on the Ohio, thereby creating a greater relative change in stage height/water levels than the dams on the UMR (White et al. 2005). The effect is that the larger, deep areas of slow-flowing water behind the dams on the lower Ohio reduce the relative amount of shallow areas in the photic zone, decreasing the effective area for benthic algal production while augmenting the volume for phytoplankton production. Our data suggests a slight shift from a benthic to phytoplanktonic production in the

Ohio after the construction of dams (Fig. 5). Reductions in benthic algae would have impacted the secondary production and diversity of benthic invertebrates, and these changes could then be responsible for species specific shifts in food sources.

Historical changes in food sources in the UMR

In contrast to the Ohio, the effects of dams on food source use in the UMR are more pronounced. Dam and levee construction on the UMR changed the river from a system of meandering channels with some lateral complexity (islands, some secondary channels) to one on a stable channel with large amounts of lateral complexity below each dam and downstream to a pool formed above the next dam downstream. This effectively increased the hydrogeomorphic complexity of the system, in-turn creating more diverse habitats for primary production and biota to thrive, leading to an increase in variability in food source use of invertivorous and piscivorous fishes following dam construction (Fig. 6; cf. Thorp et al. 2006, 2008). Although this did not change average overall use of algae from before to after dam construction (Fig. 7), there were species specific shifts in basal sources (Fig. 8).

Conclusions

The use of AA-CSIA to determine food sources used by consumers in ecosystems as well as other aspects of food webs (i.e. trophic position and food chain length; Bowes & Thorp 2015) is an exceedingly exciting prospect, and represents a huge leap forward in the field of ecology and stable isotopes. Furthermore, this study serves as a model of retrospective analyses of food webs and potential long-term environmental perturbations in river ecosystems. Additionally, the results of this study could serve as a restoration “bench mark” for the pre-dammed state of large

river food webs. The current emphasis on restoring river habitat structure—without explicitly considering food webs—has been less successful than hoped in terms of enhancing the status of targeted species and often overlooks important constraints on ecologically effective restoration.

Figures and Figure Legends

Figure 1. The $\delta^{13}C$ values of individual amino acids for each food source taxon, relative to their respective mean $\delta^{13}C$. Error bars indicate ±SD of each taxon.

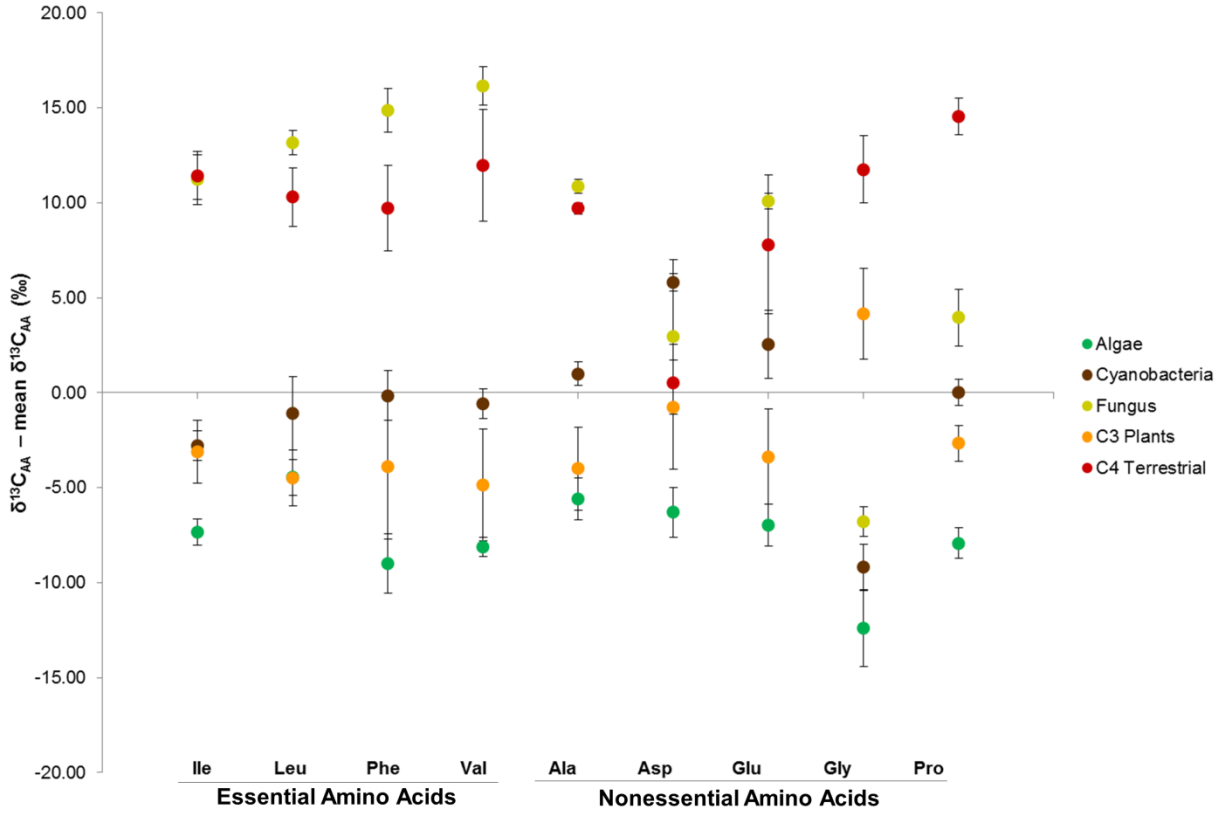

Figure 5-1 Normalized amino acids signatures for each food source taxon.

Figure 2. Principle component analysis of food sources using $\delta^{13}C$ variations among four essential amino acids (ile, leu, phe, val) and five nonessential amino acids (ala, asp, glu, gly, pro). Five distinct food source groups were present as demonstrated with the first and second principle components.

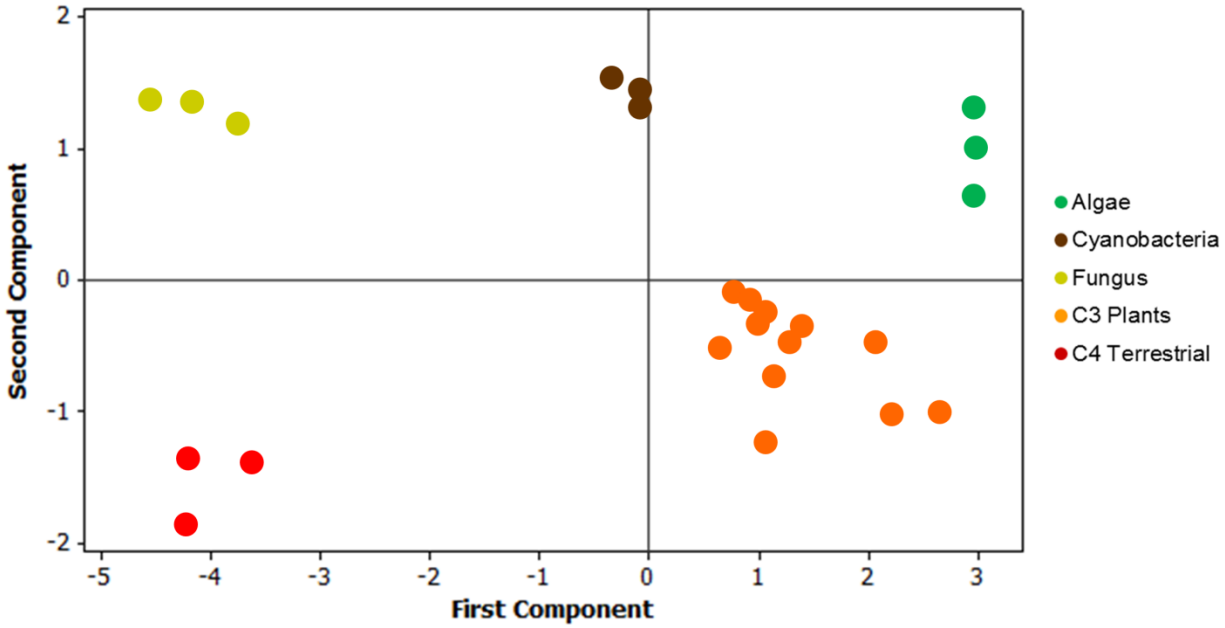

Figure 5-2 Principle component analysis of food sources.

Figure 3. Reliance of fish (combined presumptive invertivores and piscivores) from the Ohio River on various food sources through time. Points are mean values estimated by the FRUITS model (based on 4 essential amino acids (ile, leu, phe, and val), 2 conditionally non-essential amino acids (gly and pro), and 3 non-essential amino acids (ala, asp, and glu), deciphering between 5 possible food sources (cyanobacteria, green algae, fungi, C_3 terrestrial and aquatic plants, and C_4 terrestrial plants)). Black bar indicates the date in which dam construction began in the portion of the river where fish were originally collected.

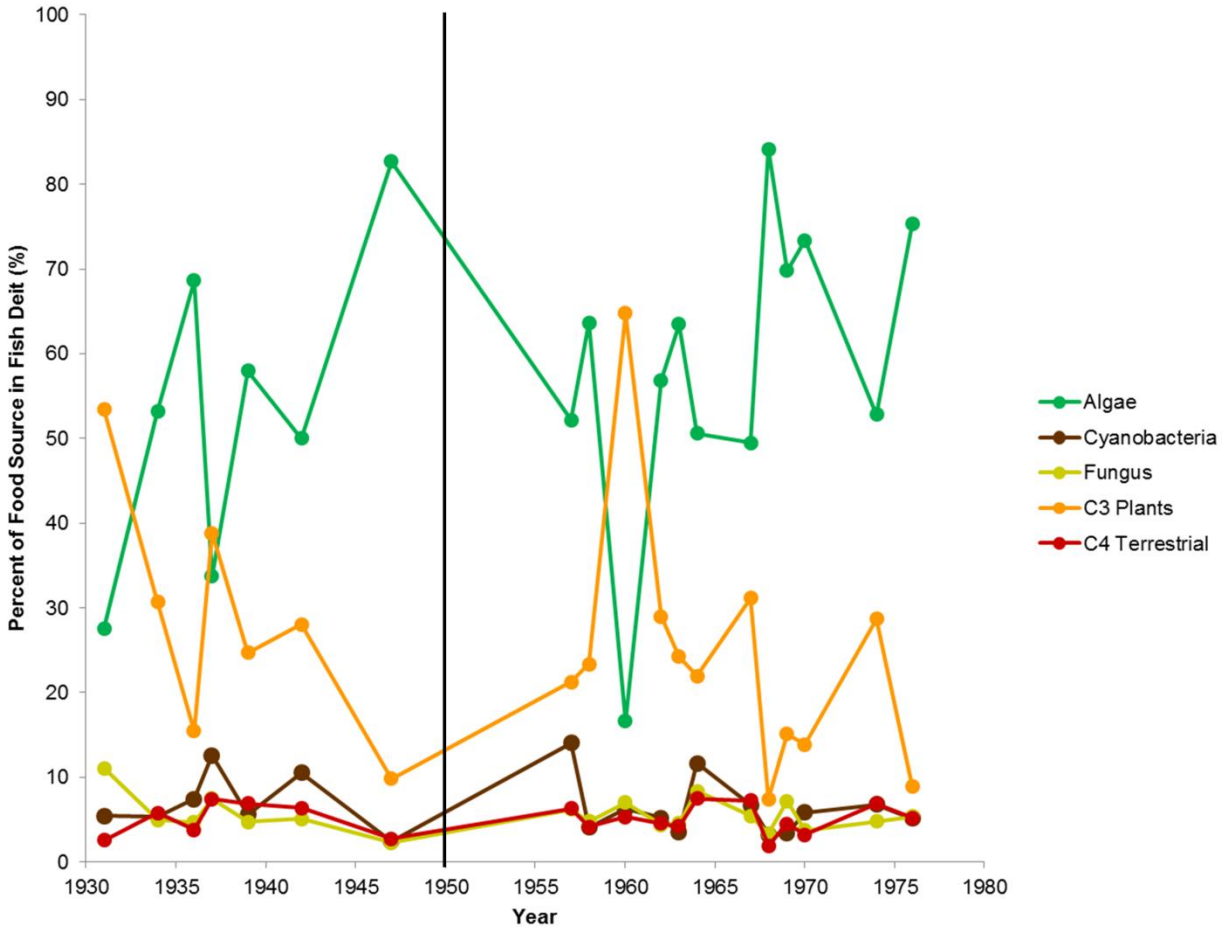

Figure 5-3 Ohio River food source changes through time.

Figure 4. Boxplots generated with FRUITS mixing model showing reliance of fish (combined presumptive invertivores and piscivores) from the Ohio River on various food sources before (a) and after (b) major dam construction. FRUITS model was based on 4 essential amino acids (ile, leu, phe, and val), 2 conditionally non-essential amino acids (gly and pro), and 3 non-essential amino acids (ala, asp, and glu), deciphering between 5 possible food sources (cyanobacteria, green algae, fungi, C_3 terrestrial and aquatic plants, and C_4 terrestrial plants). Solid bar in center of box is the mean; upper and lower bounds of box are the 68% confidence intervals; and whiskers are 95% confidence interval.

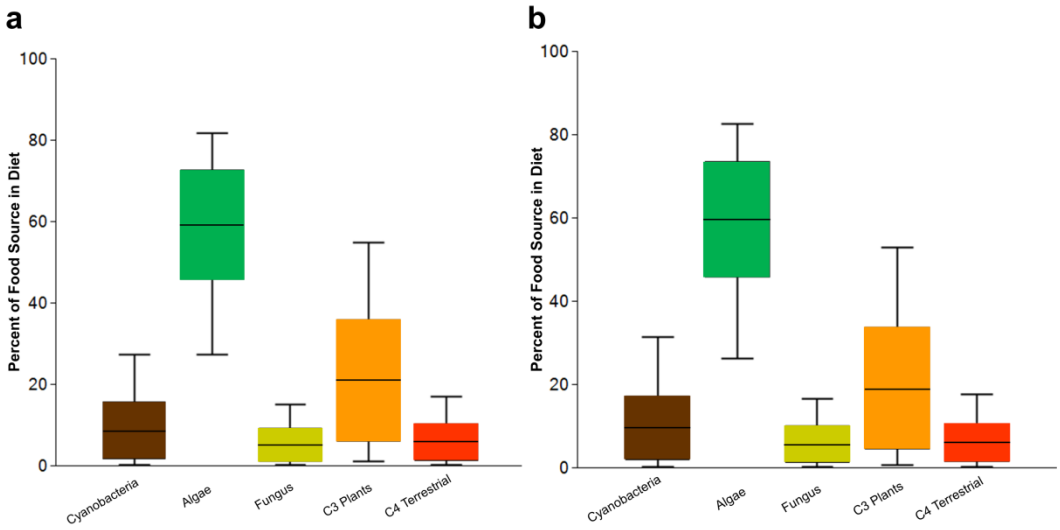

Figure 5-4 Ohio River food source before and after dams.

Figure 5. Fish in the Ohio River before (a) and after (b) major dam construction. Plots consist of trophic position versus percent algae contribution to diet as evaluated by AA-CSIA and FRUITS ($\delta^{15}N$ and $\delta^{13}C$). Each point represents a species' mean value, with error bars removed for simplicity. Color shows percent C_3 plant contribution to the diet. It is possible to see species specific shifts in diet before and after dam construction.

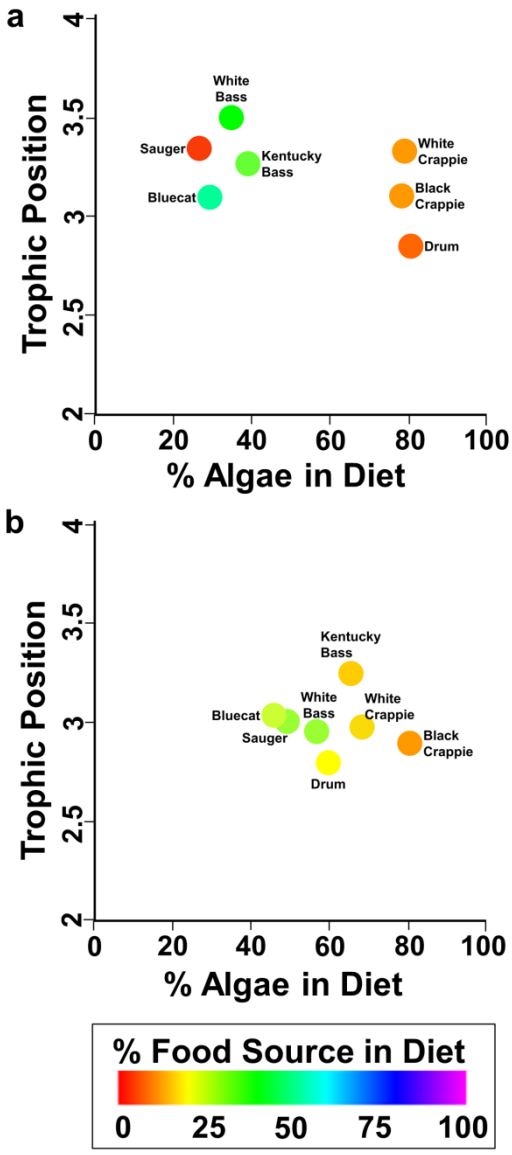

Figure 5-5 Ohio River species specific shifts.

Figure 6. Reliance of fish (combined presumptive invertivores and piscivores) from the Upper Mississippi River on various food sources through time. Points are mean values estimated by the FRUITS model (based on 4 essential amino acids (ile, leu, phe, and val), 2 conditionally non-essential amino acids (gly and pro), and 3 non-essential amino acids (ala, asp, and glu), deciphering between 5 possible food sources (cyanobacteria, green algae, fungi, C_3 terrestrial and aquatic plants, and C_4 terrestrial plants)). Black bar indicates the date in which dam construction began in the portion of the river where fish were originally collected.

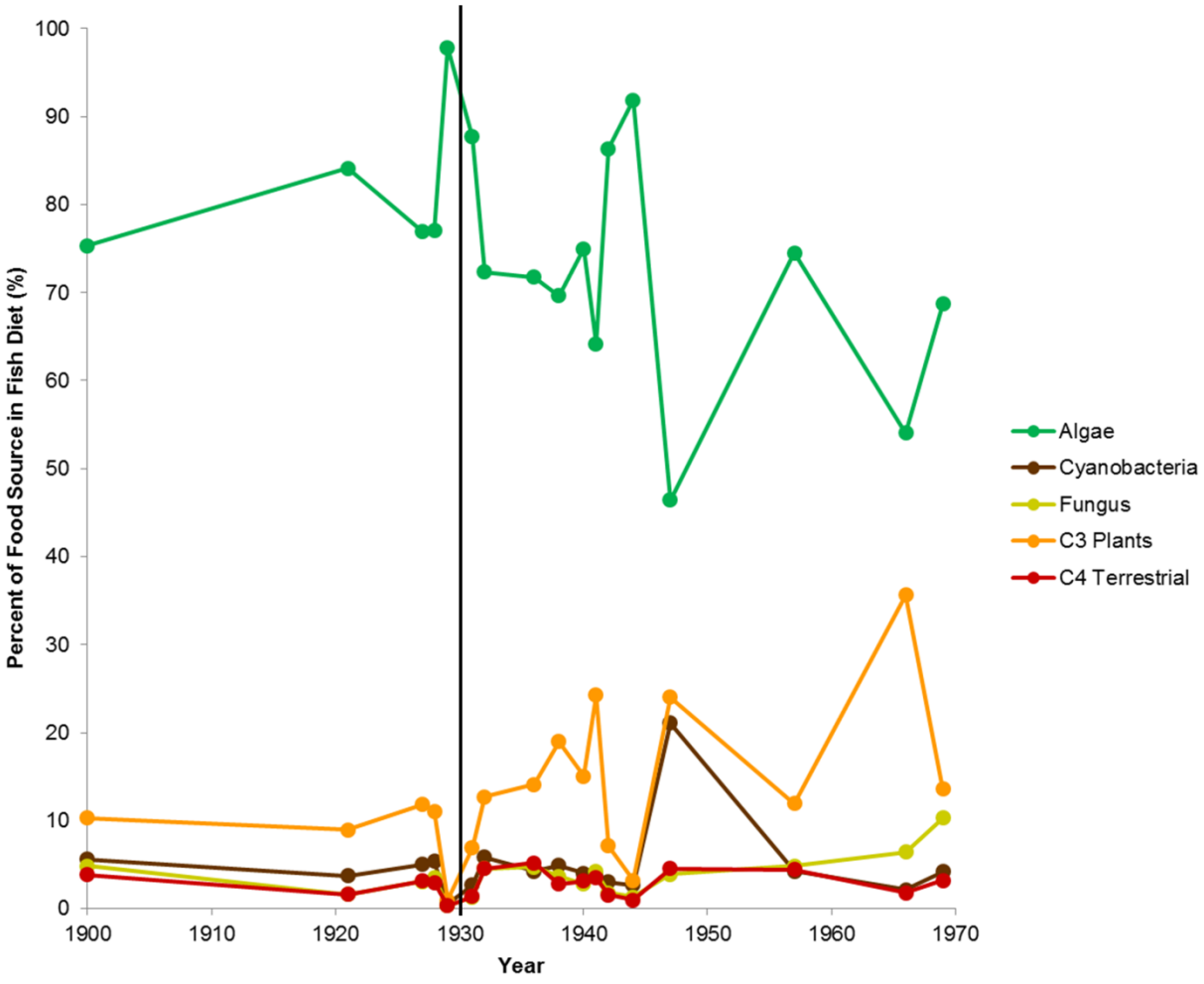

Figure 5-6 UMR food source changes through time.

Figure 7. Boxplots generated with FRUITS mixing model showing reliance of fish (combined presumptive invertivores and piscivores) from the Upper Mississippi River on various food sources before (a) and after (b) major dam construction. FRUITS model was based on 4 essential amino acids (ile, leu, phe, and val), 2 conditionally non-essential amino acids (gly and pro), and 3 non-essential amino acids (ala, asp, and glu), deciphering between 5 possible food sources (cyanobacteria, green algae, fungi, C_3 terrestrial and aquatic plants, and C_4 terrestrial plants. Solid bar in center of box is the mean; upper and lower bounds of box are the 68% confidence intervals; and whiskers are 95% confidence interval.

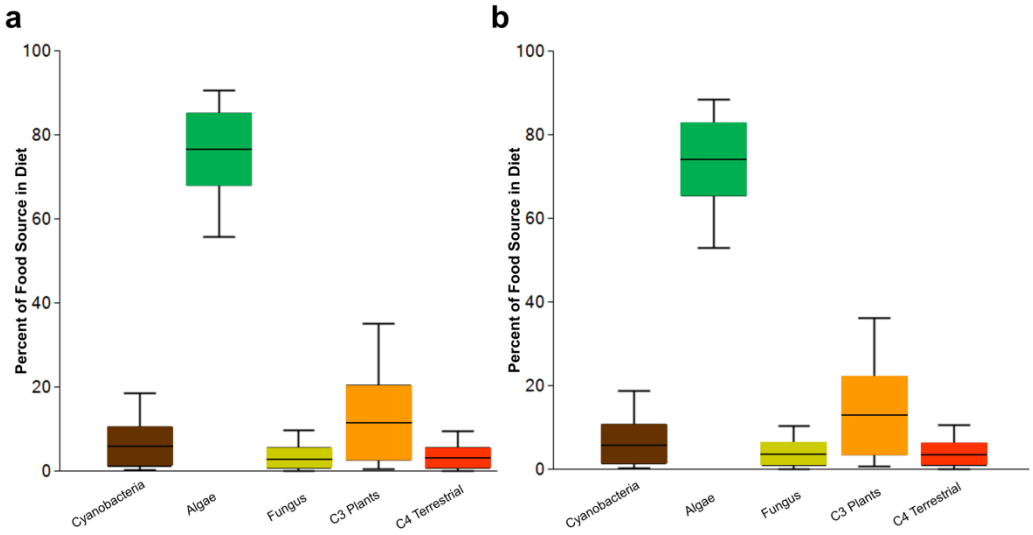

Figure 5-7 UMR food source before and after dams.

Figure 8. Fish in the Upper Mississippi River before (a) and after (b) major dam construction. Plots consist of trophic position versus percent algae contribution to diet, evaluated by AA-CSIA and FRUITS ($\delta^{15}N$ and $\delta^{13}C$). Each point represents a species' mean value, with error bars removed for simplicity. Color shows percent C_3 plant contribution to the diet. It is possible to see species specific shifts in diet before and after dam construction.

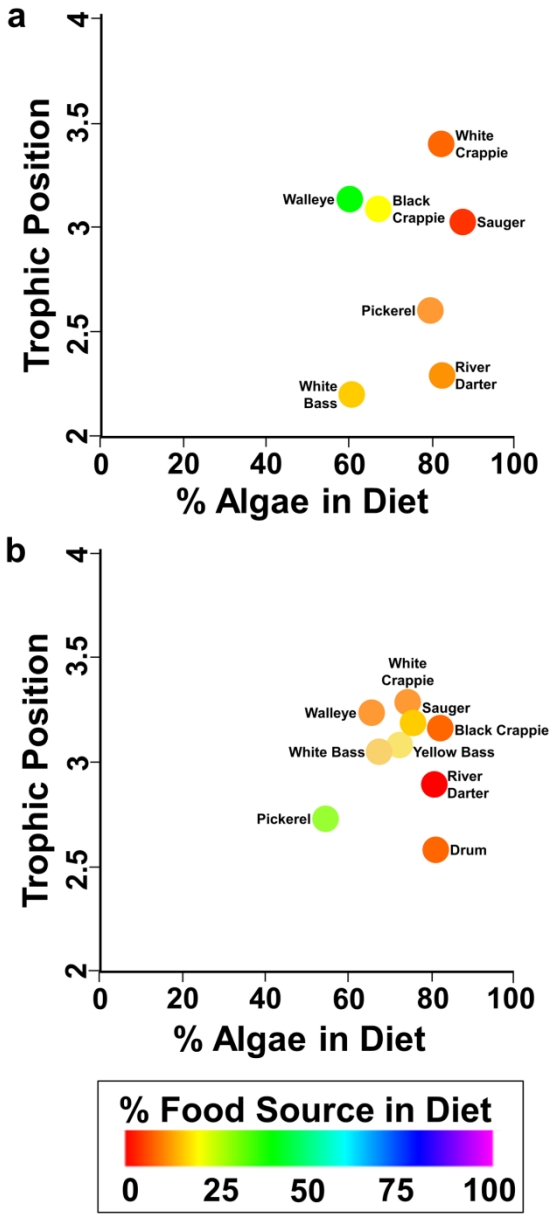

Figure 5-8 UMR species specific shifts.

Tables

Table 1. Stable isotope ($\delta^{13}C$) values for 4 essential and 5 non-essential amino acids in potential food sources for fishes in the UMR and LOR. Data represents mean $\delta^{13}C$ values ± 1 S.E., based on 3 replicates of each genus sampled. Essential Amino Acids are ile = isoleucine, leu = leucine, phe = phenylalanine, and val = valine. Conditionally Non-Essential Amino acids are gly = glycine and pro = proline. Non-Essential Amino Acids: ala = alanine, asp = aspartic acid, and glu = glutamic acid. Food sources are CYA = cyanobacteria, GRE = green algae, FUN =fungi, C_3 = C_3 terrestrial grass, tree leaves, soybean, and aquatic macrophyte, C_4 = C_4 terrestrial corn.

Food Source	Genus	Essential Amino Acids				Conditionally Non-Essential		Definitely Non-Essential		
		ile	leu	phe	val	gly	pro	ala	asp	glu
CYA	*Spirulina*	-26.74 ± 0.46	-31.05 ± 1.11	-26.35 ± 0.75	-28.07 ± 0.44	-17.83 ± 0.71	-16.37 ± 0.40	-17.85 ± 0.36	-09.20 ± 0.26	-19.75 ± 1.04
GRE	*Chlorella*	-31.29 ± 0.40	-34.42 ± 0.54	-35.17 ± 0.90	-35.62 ± 0.29	-21.04 ± 1.16	-24.30 ± 0.46	-24.44 ± 0.63	-21.33 ± 0.76	-29.24 ± 0.64
FUN	*Saccharomyces*	-12.75 ± 0.75	-16.78 ± 0.37	-11.34 ± 0.66	-11.33 ± 0.59	-15.43 ± 0.45	-12.43 ± 0.87	-07.98 ± 0.22	-12.08 ± 2.36	-12.20 ± 0.24
C_3	*Elymu, Populus, Glycine, Valisneria*	-27.06 ± 0.48	-34.45 ± 0.42	-30.07 ± 1.11	-32.36 ± 0.85	-04.47 ± 0.69	-19.05 ± 0.27	-22.85 ± 0.63	-15.77 ± 0.95	-25.67 ±0.72
C_4	*Zea*	-12.69 ± 0.58	-19.66 ± 0.90	-16.47 ± 1.29	-15.51 ± 1.69	-03.13 ± 1.02	-01.84 ±0.55	-09.15 ± 0.16	-14.52 ± 0.71	-14.48 ± 2.10

Table 5-1 Carbon isotope signatures of river food sources.

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Appendix 1

R code for metrics and confidence intervals.

```
#This code implements metrics of Layman et al (2007), Ecology, 88, 42-48, as well as
#Manhattan distance modifications of some of those metrics.
#
#Daniel C Reuman
#2015 01 21

require(geometry)

#***
#Layman metrics exactly
#***

#Ranges
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#          columns containing isotope information for each species.
#rangenum   The numbers of the isotopes (in the order of the columns in iso) for
#          which you want the range. Defaults to all of them.
#
#Output
#A vector of the same length as rangenum that gives all the ranges desired.
#
#Notes
#It is assumed that no data are missing.
#
RGS.lay<-function(iso,rangenum)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
  {
    iso<-iso[,2:dim(iso)[2]]
  }
  m<-apply(X=iso,FUN=min,MARGIN=2) #the min of each column
  M<-apply(X=iso,FUN=max,MARGIN=2) #the max of each column
  return(M[rangenum]-m[rangenum])
}

#Convex hull area/volume
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
```

```
#           columns containing isotope information for each species.
#
#Output
#A single number which is the area of the convex hull
#
#Notes
#It is assumed that no data are missing. THIS CODE IS JUST A WRAPPER AROUND
convhulln
#FROM geometry package, WHICH IN TURN CALLS THE Qhull LIBRARY. THE Qhull code
CRASHES
#IF THE CONVEX HULL HAS ZERO VOLUME.
#
CHV.lay<-function(iso)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
  {
    iso<-iso[,2:dim(iso)[2]]
  }
  res<-convhulln(iso,options='FA')
  return(res$vol)
}

#Mean distance to centroid
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#           columns containing isotope information for each species.
#
#Output
#A single number with is the mean Euclidean distance of species as points in
#isotope space to the centroid point.
#
#Notes
#It is assumed that no data are missing.
#
CD.lay<-function(iso)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
  {
    iso<-iso[,2:dim(iso)[2]]
  }
  di<-dim(iso)
  c<-apply(X=iso,FUN=mean,MARGIN=2) #the centroid
  res<-mean(sqrt(apply(X=(iso-
matrix(rep(c,times=di[1]),di[1],di[2],byrow=T))^2,MARGIN=1,FUN=sum)))
  return(res)
```

```
}

#Mean nearest neighbor distance
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#           columns containing isotope information for each species.
#
#Output
#A single number which is the mean Euclidean distance of species as points in
#isotope space to their nearest neighbors.
#
#Notes
#It is assumed that no data are missing.
#
NND.lay<-function(iso)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
  {
    iso<-iso[,2:dim(iso)[2]]
  }

  #find all the pairwise distances
  res<-0
  for (c1 in 1:dim(iso)[2])
  {
    res<-res+outer(iso[,c1],iso[,c1],FUN=function(x,y){(x-y)^2})
  }
  res<-sqrt(res)

  #get column mins
  diag(res)<-NA
  res<-apply(X=res,MARGIN=2,FUN=min,na.rm=T)

  #return the mean
  return(mean(res))
}

#Standard deviation of nearest neighbor distance
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#           columns containing isotope information for each species.
#
#Output
#A single number which is the standard deviation of Euclidean distances of
```

```
#species as points in isotope space to their nearest neighbors.
#
#Notes
#It is assumed that no data are missing.
#
SDNND.lay<-function(iso)
{
 if (names(iso)[1]=="species" || names(iso)[1]=="Species")
 {
  iso<-iso[,2:dim(iso)[2]]
 }

 #find all the pairwise distances
 res<-0
 for (c1 in 1:dim(iso)[2])
 {
  res<-res+outer(iso[,c1],iso[,c1],FUN=function(x,y){(x-y)^2})
 }
 res<-sqrt(res)

 #get column mins
 diag(res)<-NA
 res<-apply(X=res,MARGIN=2,FUN=min,na.rm=T)

 #return the standard deviation
 return(sd(res))
}

#***
#Manhattan modifications
#***

#Ranges
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#          columns containing isotope information for each species.
#rangenum   The numbers of the isotopes (in the order of the columns in iso) for
#          which you want the range. Defaults to all of them.
#
#Output
#A vector of the same length as rangenum that gives all the ranges desired.
#
#Notes
#It is assumed that no data are missing. This is identical to RGS.lay above.
#
```

```
RGS.man<-function(iso,rangenum)
{
 if (names(iso)[1]=="species" || names(iso)[1]=="Species")
 {
  iso<-iso[,2:dim(iso)[2]]
 }
 m<-apply(X=iso,FUN=min,MARGIN=2) #the min of each column
 M<-apply(X=iso,FUN=max,MARGIN=2) #the max of each column
 return(M[rangenum]-m[rangenum])
}

#Mean distance to centroid. The only modification from Layman et al (2007) is
#that we use the Mahattan distance instead of the Euclidean distance.
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#          columns containing isotope information for each species.
#
#Output
#A single number with is the mean Manhattan distance of species as points in
#isotope space to the centroid point.
#
#Notes
#It is assumed that no data are missing.
#
CD.man<-function(iso)
{
 if (names(iso)[1]=="species" || names(iso)[1]=="Species")
 {
  iso<-iso[,2:dim(iso)[2]]
 }
 di<-dim(iso)
 c<-apply(X=iso,FUN=mean,MARGIN=2) #the centroid
 res<-mean(apply(X=abs(iso-
matrix(rep(c,times=di[1]),di[1],di[2],byrow=T)),MARGIN=1,FUN=sum))
 return(res)
}

#Mean nearest neighbor distance. The only modification from Layman et al (2007) is that
#we use a Manhattan distance instead of Euclidean distance.
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#          columns containing isotope information for each species.
#
#Output
```

```
#A single number which is the mean Manhattan distance of species as points in
#isotope space to their nearest neighbors.
#
#Notes
#It is assumed that no data are missing.
#
NND.man<-function(iso)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
  {
    iso<-iso[,2:dim(iso)[2]]
  }

  #find all the pairwise Manhattan distances
  di2<-dim(iso)[2]
  res<-0
  for (c1 in 1:di2)
  {
    res<-res+outer(iso[,c1],iso[,c1],FUN=function(x,y){abs(x-y)})
  }

  #get column mins
  diag(res)<-NA
  res<-apply(X=res,MARGIN=2,FUN=min,na.rm=T)

  #return the mean
  return(mean(res))
}

#Standard deviation of nearest neighbor distance. The modification from Layman
#et al (2007) is that we use Manhattan distance instead of Euclidean distance.
#
#Arguments
#iso        A data frame which may have taxon names in the first column and subsequent
#           columns containing isotope information for each species.
#
#Output
#A single number which is the sd Manhattan distance of species as points in
#isotope space to their nearest neighbors.
#
#Notes
#It is assumed that no data are missing.
#
SDNND.man<-function(iso)
{
  if (names(iso)[1]=="species" || names(iso)[1]=="Species")
```

```
 {
  iso<-iso[,2:dim(iso)[2]]
 }

 #find all the pairwise Manattan distances
 di2<-dim(iso)[2]
 res<-0
 for (c1 in 1:di2)
 {
  res<-res+outer(iso[,c1],iso[,c1],FUN=function(x,y){abs(x-y)})
 }

 #get column mins
 diag(res)<-NA
 res<-apply(X=res,MARGIN=2,FUN=min,na.rm=T)

 #return the mean
 return(sd(res))
}

#***
#Resampling code
#***

#Code for resampling-based confidence intervals for the above metrics
#in the event that individual-level data are available. Resamples
#individuals within species. There should be at least 2 and probably
#3 or more individuals per species available.
#
#Arguments
#iso.indiv  A data frame which has taxon names in the first column and subsequent
#          columns containing isotope information for each individual measured
#          of each species. There should me multiple rows for each taxon name,
#          indistinguishable except they will typically have different values
#          for the isotope measurements of the different individuals. The first
#          column *must* contain taxon names, and must be titled either "species"
#          or "Species".
#func      One of the metric functions above
#func.args  Additional arguments to func - NA for all metrics except the range
#          functions
#numresamp  Number of resamplings desired, more means more accurate confidence
#          intervals. Use 10000 for final runs, 100 for debugging.
#
#Output
#A list with elements as follows:
#pt.est     The output of func on the real data - should be the same as calling
```

```
#          the metric function directly
#dist      The output of func on the resamplings. Has as many entries as numresamp,
#          you compute quantiles using the R function "quantile" to get confidence
#          intervals.
#
#Notes
#It is assumed that no data are missing.
#
resamp.indiv<-function(iso.indiv,func,func.arg=NA,numresamp=1000)
{
 #a bit of error checking to make sure each species has enough individuals
 if (names(iso.indiv)[1]=="Species")
 {
  names(iso.indiv)[1]<-"species"
 }
 for (sp in unique(iso.indiv$species))
 {
  if (length(iso.indiv$species[iso.indiv$species==sp])==1)
  {
   stop("Error in resamp.indiv: you need at least 2 samples per individual, preferably 3 or
more.")
  }
 }

 #make iso.indiv into iso by computing species means
 iso<-aggregate(iso.indiv[,-1],list(iso.indiv$species),function(x){mean(x,na.rm=T)})
 names(iso)[1]<-"species"

 #call func to get the point estimate
 if (is.na(func.arg))
 {
  pt.est<-unname(func(iso))
 } else
 {
  pt.est<-unname(func(iso,func.arg))
 }

 #iteratively resample from iso.indiv, re-mean to get
 #surrogate iso data frames, and recompute metric values
 dist<-matrix(NA,numresamp,length(pt.est))
 for (counter in 1:numresamp)
 {
  #resample
  iso.indiv.r<-iso.indiv
  for (sp in unique(iso.indiv$species))
  {
```

```
    h<-iso.indiv.r[iso.indiv.r$species==sp,]
    dh<-dim(h)[1]
    h<-h[sample(1:dh,dh,replace=T),]
    iso.indiv.r[iso.indiv.r$species==sp,]<-h
   }

   #re-mean
   iso<-aggregate(iso.indiv.r[,-1],list(iso.indiv.r$species),function(x){mean(x,na.rm=T)})
   names(iso)[1]<-"species"
   if (any(is.na(iso))){next}

   #call the function and save result
   if (is.na(func.arg))
   {
    r.est<-unname(func(iso))
   } else
   {
    r.est<-unname(func(iso,func.arg))
   }
   dist[counter,]<-r.est
  }

  return(list(pt.est=pt.est,dist=dist))
}

#Code for resampling-based confidence intervals for the above metrics
#in the event that species-level data are all that is available, but with
#standard errors. Resamples values from normal distributions with the given
#standard error, but subject to provided bounds.
#
#Arguments
#iso        A data frame which has taxon names in the first column and subsequent
#          columns containing isotope information for each species.
#se         Same format as iso, but with the corresponding standard errors.
#lobds      One entry for each column of iso except the first, providing lower
#          bounds of possibility for values in iso, for resampling. For instance,
#          values which are percentages of diet should be bounded below by 0.
#          Use -Inf if no bound.
#hibds      Same, but upper bounds
#func       One of the functions above
#func.args  Additional arguments to func
#numresamp  Number of resamplings desired
#
#Output
#A list with elements as follows:
#pt.est     The output of func on the real data
```

```
#dist      The output of func on the resamplings
#
#Notes
#It is assumed that no data are missing.
#
resamp.se<-function(iso,se,lobds,hibds,func,func.arg=NA,numresamp=1000)
{
 #call func to get the point estimate
 if (is.na(func.arg))
 {
  pt.est<-unname(func(iso))
 } else
 {
  pt.est<-unname(func(iso,func.arg))
 }

 #iteratively resample from iso, and recompute metric values
 dist<-matrix(NA,numresamp,length(pt.est))
 lobds<-matrix(lobds,dim(iso)[1],length(lobds),byrow=T)
 hibds<-matrix(hibds,dim(iso)[1],length(hibds),byrow=T)
 for (counter in 1:numresamp)
 {
  #resample
  iso.r<-iso
  iso.r[,-1]<-rnorm(prod(dim(iso[,-1])),as.matrix(iso[,-1]),as.matrix(se[,-1]))
  iso.r[,-1]<-pmax(as.matrix(iso.r[,-1]),lobds)
  iso.r[,-1]<-pmin(as.matrix(iso.r[,-1]),hibds)

  #call the function and save result
  if (is.na(func.arg))
  {
   r.est<-unname(func(iso.r))
  } else
  {
   r.est<-unname(func(iso.r,func.arg))
  }
  dist[counter,]<-r.est
 }

 return(list(pt.est=pt.est,dist=dist))
}
```