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AN ISOTOPIC EXAMINATION
OF CAVE, SPRING AND EPIGEAN
TROPHIC STRUCTURES IN MAMMOTH
CAVE NATIONAL PARK

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AN ISOTOPIC EXAMINATION
OF CAVE, SPRING AND EPIGEAN
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Abstract

High-water events in the Green River result in flow-reversals which flush native and introduced fishes into Mammoth Cave, posing threats to indigenous cave fauna. However, little is known about the trophic interactions between cave and epigean aquatic systems or their connectivity via natural springs. The purpose of this study was to use stable isotopes of C and N to describe and compare the trophic structure of epigean, spring and cave aquatic systems within Mammoth Cave National Park. Fourteen sites were sampled from fall 2002 to fall 2003; four in the Green River (epigean), four in spring-heads, and three inside Mammoth Cave. Two *a priori* hypotheses were tested: fish and invertebrates living in spring heads should express $\delta^{13}C$ values intermediate to those of organisms in cave and epigean aquatic systems and overall trophic levels in cave and spring samples should be compressed, showing lower $\delta^{15}N$ values compared to epigean sites. Though cave and spring systems were dominated by allochthonous leaf litter, characteristic of headwater streams, the epigean system was also largely dependent on detrital inputs. Primary differences in $\delta^{13}C$ were seen at higher trophic levels, particularly in top consumers such as *Lepomis* species, where $\delta^{13}C$ values decreased from epigean to

spring to cave habitats. Though all three habitats supported a similar number of trophic levels (N: 5), the trophic structure was compressed in cave and spring compared to epigean habitats. This trend, however, was obfuscated by δ^{15}N values of accidental species in caves, which tended to be enriched, even when compared to epigean signals. This was attributed to either trophic enrichment from yolk sacs or starvation and subsequent self-processing. Overall, spring trophic structure was found to be intermediate to cave and epigean trophic structures in terms of δ^{13}C values of upper-level fish consumers, but spring trophic structure was more similar to the cave trophic structure in terms of δ^{15}N values, excluding cave accidentals.

Introduction

The relationship between trophic structure and ecological energetics appears simple until an attempt is made to establish cause and effect (Hairtson and Hairtson 1992). Traditionally, efforts at modeling trophic interactions have taken one of three approaches: 1) food-web studies seeking consistent patterns of predation among community members; 2) effect studies attempting to determine factors structuring communities; and 3) flow studies concerned with transfer of energy, nutrients, and contaminants through ecosystems (Vander Zanden and Rasmussen 1996).

Stable isotope ratios provide valuable insight into identifying and quantifying trophic pathways and processes in both field and laboratory situations (Conway et al. 1989). The utility of stable isotope analysis comes from the highly predictable alteration of isotope ratios by both biological and non-biological processes (Peterson and Fry 1987). Well-characterized key, or root, reactions are responsible for the isotopic composition of most organic matter, which is often passed through trophic pathways with minute and predictable changes. These changes are most often expressed in terms of del (δ) values, which are parts per thousand differences from a standard:

$$\delta X = \{(Rsample\ /\ Rstandard) - 1\} \times 10^3, \qquad (1)$$

where X is ^{13}C, ^{15}N, or ^{34}S, and R is the corresponding ratio of $^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{34}S/^{32}S$ (Peterson and Fry 1987). Standards can potentially include any known reference materials, although typical references include carbon in the PeeDee limestone, atmospheric nitrogen gas and sulfur from the Canyon Diablo meteorite (Peterson and Fry 1987).

δ values indicate the amounts of heavy and light isotopes in a sample: increases in δ values represent increases in heavy isotope content (^{13}C, ^{15}N or ^{34}S), whereas decreases represent an increase in light isotope content (^{12}C, ^{14}N or ^{32}S). Root reactions alter, or "fractionate," stable isotope ratios, often by very small but detectable amounts. A large change of 10% between reactants and products involves only minute absolute changes of 0.04%, 0.11%, and 0.44% for the respective heavy isotopes of nitrogen, carbon and sulfur, necessitating the use of a mass spectrometer employing precision of ±0.02% or better (Peterson and Fry 1987). Therefore, isotope analysis provides information into both the origins of certain elements—that is, where the base food source ultimately comes from (Rau 1981, Rau et al. 1983, Fry and Sherr 1984, Rounick and Winterbourne 1986, Spiro et al. 1986)—and the trophic level of an organism with a diet of isotopically distinct food sources (Fry and Sherr 1984).

Ratios of carbon isotopes can be used to separate food web components (Rounick and Winterbourn 1986, Peterson and Fry 1987, Kennicutt et al. 1992, France and Peters 1997), whereas nitrogen isotopes are more useful in determining the trophic level of organisms in the food-web (DeNiro and Epstein 1981, Minagawa and Wada 1984, Peterson and Fry 1987). This is because carbon remains relatively unchanged between successive trophic levels (^{13}C is enriched an average of 1δ each trophic level) while nitrogen demonstrates a much more noted change between trophic levels (^{15}N enrichment averages 3.4δ as it moves up each trophic level) (Colaco et al. 2002).

Despite the conservative nature of carbon as it passes up the food web, the δ values of autotrophs usually vary greatly between aquatic and terrestrial primary

producers and can be used to differentiate allochthonous from autochthonous carbon

(Hershey and Peterson 1996, although see Lazerte and Szalados 1982, France 1995a,

1996a for exceptions). Nitrogen δ values of primary producers, however, are usually 0,

with the ratio of isotopes very close to the standard. Each time the material is processed

through a successive trophic level, the nitrogen ratio increases by approximately 3.4

δ units. Consequently, isotope studies have implemented multiple isotope markers,

enabling the discrimination of specific sources of nutrition for food-web components

(Sullivan and Moncreiff 1990, Hamilton et al. 1992).

One such problem results because stomach contents represent food consumed

Until the implementation of isotope analysis, the primary method of estimating

energetic and trophic aspects of the food web was analysis of stomach contents. This is

done very roughly by identifying and counting complete or fragmented parts of organisms

in the stomach contents. Therefore, traditional gut-content analysis has several

disadvantages.

One such problem results because stomach contents represent food consumed

over a small time period, within a confined area, leading to results that do not concretely

demonstrate whether food partitioning is the exception or the rule (Bootsma et al. 1996).

This is true especially for fish (Vander Zanden and Rasmussen 1996), where reliable

averages incorporating spatial and temporal variation cost considerable time and effort as

well as high numbers of sacrificed fish (Winemiller 1990). Moreover, stomach analyses

are messy and present difficulties in identifying and determining whether all observed

stomach contents are digested to the same degree, or if some components, such as

cyanobacteria, prove indigestible (Ribbink et al. 1983, Reinthal 1990).

Another problem with traditional gut-content analysis is that often there is a lack of specific data on trophic interactions to give insight into the complexity of the trophic model. Rather, assumptions are often made of one-to-one, direct trophic relationships. This is problematic because trophic position models must then assume trophic position of lower-level species. Vander Zanden and Rasmussen (1996) found their trophic position model problematic because it assumed discrete trophic levels of invertebrates, many which have been found to be omnivorous, with a wide-ranging diet including detritus, primary producers, herbivorous zooplankton, and even predatory zooplankton species (Cooper and Goldman 1980, Grossnickle 1982). A simplified representation of these lower trophic levels ignores the complexity of detrital and microbial food webs so important to lake and river ecosystems (Wetzel 1995). Analysis of interactions at lower levels is further complicated by the fact that many invertebrates do not consume hard food parts, causing discrepancies between organisms identified in stomach contents and assimilated material (Vander Zanden and Rasmussen 1996).

Many of the problems with conventional gut-content methods can be avoided with the implementation of stable isotopes in food web studies (DeNiro and Epstein 1981, Vander Zanden and Rasmussen 1996). One advantage of stable isotopes is its inherent sampling simplicity, which is very important to limnologists and aquatic field biologists. The isolated nature of many aquatic systems (e.g. hydrothermal vents, remote glacial lakes, deep cave streams) in addition to adverse and unpredictable weather patterns often makes frequent, systematic sampling very difficult. Isolated springs, for example, can prove very inaccessible, often occurring at the bottom of large lakes or exiting a cave.

Additionally, stable isotopes have been shown to elucidate ecological structure (Haines 1976, Fry et al. 1978, Peterson et al. 1985, Wada et al. 1987).

Another advantage of stable isotopes over traditional methods is the conservative number of samples required for trophic elucidation. Often species in these remote locations are rare or endangered. In many cases it may be ecologically harmful to sample a given habitat with the thoroughness required for traditional approaches. To obtain reliable averages integrating temporal and spatial variation in a fish community requires the sacrifice of many fish, not to mention the investment of much time and effort (Trippel and Beamish 1993). In the case of a remote cave spring, for instance, thorough sampling could devastate a local ecosystem by depleting the fish community. Isotope analysis avoids these problems because samples are very small. Thousandths of a gram of tissue are all that are required to perform most analyses, which equates to only a few macroinvertebrates. In fish, the impact is even less because all that is required is a fin clip of approximately two square centimeters, preventing the sacrifice of individuals.

A final advantage of isotopes is the vast spatio-temporal implications of the data. Use of isotope ratios provides a continuous, time-integrated, quantitative measure of relative trophic position. Since isotope ratios do not require assumptions about prey trophic levels, they have been used to resolve such issues as pelagic trophic structure and omnivory which have traditionally complicated gut-content analyses (Cabana and Rasmussen1994, Gu et al. 1994). They are also good for comparative studies, such as discriminating between realized and potential trophic structure (Kling et al. 1992). Consequently, isotope analysis serves as a more accurate alternative to diet data in

resolving trophic position, so long as variation in primary producers is taken into

consideration (Yoshioka et al. 1994).

Contemporary Uses of Isotopes:

Based on their inherent advantages, three dominant modes of study persist in

contemporary research: 1) ecological monitoring (see Peterson et al. 1993, Norman et al.

1995, Vander Zanden and Rasmussen 1996), 2) assessing trophic relations of organisms

found in remote and/or pristine ecosystems (see Mizutani and Wada 1988, Conway et al.

1989, Dover and Fry 1994), and 3) resolving subtle differences in complex, non-linear

trophic systems (see France 1995a, Bootsma et al. 1996). This latter category has been

the most prolific and problematic. France (1995a) was able to differentiate between the

subtle differences separating littoral and pelagic food webs in four Canadian Shield lakes.

Other studies have focused on the subtle differences of inter-specific food partitioning.

Bootsma et al. (1996) found that inter-specific differences in isotopic composition imply

that species using similar food types occupy different habitats, suggesting that species

occupying the same habitat must utilize different food types in order to have different

isotopic compositions.

The use of isotopic analyses in elucidating more complicated pathways of food

source provenance is problematic. ^{13}C discrimination of attached algae, for instance, has

been shown to be influenced by such factors as water turbulence (France 1995b) and

macrophytes (Osmond et al. 1981). Depending on these confounding influences,

autochthonous and allochthonous δ^{13}C values may be either similar or widely divergent

(Lazerte and Szalados 1982, France 1995c, 1996a). Likewise, in complex ecotonal food webs, δ^{15}N loses its strength as an inviolate marker of ultimate trophic position (France 1994, 1995d). Often δ^{15}N in freshwater food webs reflects the combination of two trophic food source influences, as seen when the differing δ^{15}N values of terrestrial and aquatic plants hybridize markings of benthic freshwater food webs (France 1995e). This results in δ^{15}N values for individual species that are almost always higher than for mixed assemblages of organisms due to the homogenization of feeding relationships in the latter case (France et al. 1996).

Consequently, many linear mixing models have been developed to estimate trophic contribution from two sources using signatures from a single element (δ^{13}C) (see Balesdent and Mariotti 1996) or for three sources using signatures for two elements (δ^{13}C and δ^{15}N) (see Phillips 2001). Often these models over-simplify systems, and many have been meet with criticism. Because of natural variability in isotopic signatures and sampling error, it has been recommended that mixing models will work best when sources are farther apart (Dawson 1993, Hogberg 1997), with the minimum distance between sources dependent primarily upon the source and mixture standard deviations, the sample size, and the width of the desired confidence interval (Phillips and Greg 2001). Other criticisms of these models involve the difficulty in establishing δ^{15}N baselines (Vander Zanden and Rasmussen 1999, Post 2002) and efforts have been made, such as using primary consumers and curve-fitting methods, to establish this important baseline (Post 2002).

After decades of work on even relatively simple trophic models such as deep-sea

vent fields, complete descriptive trophic models are only now being elucidated and explored (see Colaco et al. 2002). Despite efforts in marine studies to relate actual organismal trophic position as measured by $\delta^{15}N$ to progressive $\delta^{13}C$ enrichment (Wada et al. 1987, Hobson and Welch 1992), *a priori* adjustments of organismal ^{13}C to accommodate trophic fractionations in freshwater food webs may be inappropriate and will only serve to further obfuscate the already complicated task of describing energy flow pathways (see France 1996b).

The notion of discrete trophic levels continues to be challenged. Such phenomena as omnivory, opportunistic feeding in fish and macroinvertebrates, and seasonal system dynamics have confounded such traditional ideas as discrete trophic "levels" or the notion of "food chains" in favor of more relative terms such as "trophic height" and "vertical foodweb structure" (see Yodzis 1984, France et. al. 1996).

Most of the isotope literature until recently has focused on single systems, neglecting to examine interactions across systems. In the last several years, for instance, there has been increasing interest in the connections between the aquatic and terrestrial systems (see Busch et al. 1992, Collier et al. 2002). Largely overlooked among aquatic systems in isotopic studies are the subterranean aquifers of caves, especially in regard to their interactions with surface systems. Subterranean systems prove complicated both because of their often remote nature and because, lacking light, they are void of primary producers and relatively depauperate. Relying exclusively from surface detrital inputs through sinkholes or sinking streams, subterranean streams represent heterotrophic end points in the continuum of stream types (Simon et al. 2003). Of the limited isotope

studies that have been done of aquatic cave systems, few have examined the interface between epigean and subterranean aquatic systems. Yet, the hydrological connection between these systems, from subterranean aquifers through springs to surface streams, reveals their potential interplay.

Thought to be extinct from 1967 to 1979, the Kentucky Cave Shrimp (*Palaemonias ganteri*) was found to be thriving in deep, base-level pools within Mammoth Cave in the early 1980s (Holsinger and Leitheuser 1982a, Holsinger and Leitheuser 1982b, Holsinger and Leitheuser 1983a, Leitheuser 1984, Lisowski 1983). Concern for the endangered shrimp brought heightened research geared toward its preservation. One of the primary issues of concern was that introduced rainbow trout (*Oncorhynchus mykiss*) were migrating down from stocking sites in the Nolin River and Lynn Camp Creek, making their way through the Green River to cave springs and preying upon rare and endangered cave fauna via these cave access points. Though there has been skepticism that rainbow trout would be found thriving in the warm summertime waters of the Green River, it was thought that individuals of this cool-water species might be making their way upstream during the cooler, high-flow seasons, only to become trapped and localized to cool cave spring heads during warm-water periods.

A sighting by Arthur T. Leitheuser (Holsinger and Leitheuser 1983b) of a rainbow trout preying upon a cave shrimp within Pike Spring of the Mammoth Cave System heightened concern. Although there is evidence from creel surveys that rainbow trout are at times abundant in the Green River (Bonnie Laflin, unpublished data), intensive sampling in 2002 and 2003 failed to collect them in the Green River (Compson and

Lienesch, unpublished data). However, preliminary sampling in the present study

indicated that many predators native to the Green River watershed get flushed into the

cave during high-water events, posing realistic, though natural, threats to cave fauna.

Due to the remote and sensitive nature of the ecosystems within the Mammoth

Cave drainage system, stable isotope techniques provide an important, minimally

invasive method of examining the largely unstudied trophic systems within Mammoth

Cave National Park (MCNP). Aside from the work conducted by Harmon (1979) using

oxygen isotopes to examine vadose seepage rates and their effects on the isotopic

composition of precipitated speleothem calcite, there have been no isotope studies

conducted within MCNP, and no study has examined trophic structures within MCNP

using stable isotopes. The purpose of this study was to utilize the isotopic ratios of δ^{13}C

and δ^{15}N in order to describe the trophic structure of epigean (i.e., surface stream) spring

and cave aquatic systems within MCNP and elucidate differences for both fish and

invertebrate species among these systems. Two *a priori* hypotheses were established at

the outset of this experiment: fish and invertebrates living in spring heads should express

δ^{13}C values intermediate to those of organisms in cave and epigean habitats and overall

trophic levels in cave and spring samples should be compressed, showing lower δ^{15}N

values compared to epigean sites.

Materials and Methods

Sampling took place in Mammoth Cave National Park from August to December,

2002, and May to August, 2003. The study included 12 sites: 4 cave sites (DS, ERP, OC

and RSS); 4 sites at spring heads exiting the cave (ER, PS, RS, and SC); and four sites along the main stem of the Green River (G1, G2, G3, and G4) (Table 1). Cave sites were selected based on their accessibility and hydrological connection to one of the four aforementioned spring sites: ERP drains into ES, RSS and DS drain into RS, and OC drains into Turnhole Bend Spring, just downstream of SC. A fifth cave site—the Golden Triangle, which drains into PS—was inaccessible due to flooding. Epigean sites were distributed along the length of the Green River inside MCNP and were chosen because they are part of MCNP's long-term monitoring program.

Fish samples were collected using three methods. Main-stem samples were collected using a boat electroshocker. Samples were taken from the spring-heads using backpack electroshockers with modified probes that could be placed across the spring heads, allowing for larger fish to be sampled. Cave samples were collected primarily using backpack electroshocker, with additional samples taken from gill-nets, minnow traps, and larval fish traps. Tissue samples were taken from the caudal fin of large fish (generally ≥ 100 cm), and the individual was released into the vicinity of its capture. Small individuals (generally < 100 cm) were killed, with tissue from pectoral and anal fins added to the caudal sample in order to provide enough tissue for analysis. In rare cases (where noted), cave samples were too small (TL < 30 mm) and the entire body of a given individual was processed for isotope analysis. However, preliminary analysis of fin, gill, gut and muscle tissues of four *Micropterus salmoides* individuals from G1 revealed no significant differences in either δ^{13}C ($F_{2,13}$: 0.302; P: 0.824) or δ^{15}N ($F_{2,13}$: 0.360; P: 0.783) among tissue types. All fish samples were rinsed to remove debris,

stored in sealed vials and dried immediately upon return to the lab.

Invertebrates were sampled using kick-nets, root jabs and rock picks. Samples were rinsed with deionized water and sorted to order within one hour of collection or refrigerated in deionized water and sorted to order within three days of collection. Invertebrate samples involving multiple individuals were pooled to attain the proper dry-mass requirements and reduce seasonal variability in isotopic composition (see Nichols and Garling 2000). Epigean crayfish (*Cambarus tenebrosus*) from the main-stem and spring sites were all obtained using kick-nets. Cave crayfish (*Orconectes pellucidus*) were primarily caught in baited minnow traps, with some also acquired in gill nets.

Additionally, moss, algae, detritus, bacteria, and water samples were taken (when available) at each of the respective main-stem, spring, and cave sites. Moss and algae samples were scraped from rocks upstream of spring-head confluences in the main-stem and within springs. Detritus was collected at all main-stem and spring sites, and two of three cave sites by manually picking it from kick-net samples. Bacterial samples were taken from the top 10-mm of sediment from main-stem, spring, and cave steam beds using a dissecting spatula and stored in 50-ml glass vials. All bacteria samples were drained and dried before being sent to the Colorado Plateau Stable Isotope Laboratory (CPSIL) at Northern Arizona University (NAU) for further processing. All samples were dried at 60 °C for 48 hours after collection. After drying, samples were pulverized and weighed into tin capsules (0.6 - 0.8 mg for animal tissue; 1.2 mg for plant tissue). A single sample for a given taxa ranged from N =1 (for fish) to N = 80 (some invertebrates), and sample numbers varied (Table 2). Samples were sent to the CPSIU at NAU and

analyzed using a Thermo Finnigan gas isotope-ratio mass spectrometer to obtain ratios for carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$). International standards were PDB (Pee Dee belemnite) carbonate and atmospheric nitrogen gas (Peterson and Fry 1987).

Statistics were conducted using SYSTAT version 9.0 (SPSS 1999). $\delta^{13}C$ and $\delta^{15}N$ values were log-transformed to normalize the data and equalize the variance, using the following two formulae:

$$\delta N_t = \ln (\delta N), \qquad\qquad\qquad\qquad (2)$$

where δN is the original nitrogen δ value and δN_t is the transformed value, and

$$\delta C_t = \ln (-(\delta C)), \qquad\qquad\qquad\qquad (3)$$

where δC is the original carbon δ value and δC_t is the transformed value.

Fish data were grouped by species and site and tested for deviations from normality by year using the Kolmogorov-Smirnov/Lilliefors algorithm in SYSTAT. Of 44 main-stem data sets for $\delta^{13}C$ and $\delta^{15}N$ values, only one (N δ-values for *Dorosoma cepedianum* in G1 in 2003) (2.3%) deviated significantly from normality (df = 4, Lilliefors P = 0.00286).

Of 34 sets of data from site-specific species groupings for spring fish, none (0%) deviated significantly from normality. Due to low sample numbers for 2002, data for *Lepomis* species (*L. megalotis* and *L. macrochirus*) were pooled for ^{13}C samples in RS and ES and ^{15}N samples in ES, with none of the three groupings (0%) deviating from normality. Additionally, values for two crayfish (*C. tenebrosus* and *O. pellucidus*) from PS were normally distributed.

Due to the low numbers of fish from any given species at the cave sites (Table 2)

only ten groups for fish and six groups for crayfish were tested for normality, with no groups (0%) deviating significantly from normality. Because of the limited evidence for non-normality, no further transformations were applied to the data.

Temporal comparisons of both $\delta^{13}C$ and $\delta^{15}N$ values for *M. punctulatus*, *L. megalotis*, and *L. macrochirus* between 2002 and 2003 were analyzed using unpaired Student t-tests with a Bonferroni-corrected critical t-value (0.0023), with only 2 of 21 individual comparisons yielding significant differences (Table 3). Based on the limited evidence for temporal differences, $\delta^{13}C$ and $\delta^{15}N$ data for each taxa were pooled temporally for all other comparisons.

Additionally, ANOVA and t-tests were conducted to make spatial comparisons within respective habitat types (cave: DS, ERP, OS, and RPP; main-stem: G1, G2, G3, and G4; and spring sites: RS, ES, SC, and PS) to determine where within habitat spatial differences existed for temporally pooled data for each species sampled (Table 4). Within the cave habitat, no differences were found among sites. Within the spring habitat, only $\delta^{13}C$ values for *M. punctulatus* were different among RS, ES, SC, and PS sites, with Bonferroni corrections revealing a lower $\delta^{13}C$ signal in ES compared to either PS (df: 25; P: 0.003) and SC (df: 25 ; P: 0.013). Within the epigean habitat, Bonferroni-corrected multiple comparisons for *M. punctulatus* revealed an enriched $\delta^{13}C$ signal for site G1 compared to all other sites (df: 24; all $P \leq 0.001$) and an enriched $\delta^{15}N$ signal in G3 compared to G4 (df: 24; P: 0.016). Bonferroni-corrected multiple comparisons for *D. cepedianum* within the epigean habitat revealed only $\delta^{15}N$ enrichment in G1 compared to G4 (df: 12; P: 0.013). Finally, an unpaired t-test using unequal variances revealed

Ambloplites rupestris from epigean habitat had trophic enrichment in δ^{15}N values in G3 compared to G4 (t: 3.659; df: 4.9; P: 0.015). Due to the limited statistical differences found among these groups, all spatial data (e.g., among habitat-specific sites) were pooled for all among habitat-type comparisons (e.g., among cave, spring, and epigean habitats).

Hypotheses were addressed using δ^{13}C and δ^{15}N data, pooled temporally by site and spatially by habitat type. Additionally, dual-plot C-N graphs were created to examine the trophic structure in cave, spring, and epigean habitats. Statistical comparisons of δ^{13}C and δ^{15}N values among habitats could only be made for the most abundant taxa, with differences among δ^{13}C and δ^{15}N values for individual taxa tested using ANOVA and Bonferroni-corrected multiple comparison tests. SYSTAT version 9.0 (SPSS 1999) uses the classic Bonferroni procedure where, given a collection of hypotheses, H_1, H_2,...,H_n, and an experiment-based error rate of α, each individual hypothesis H_i is tested at a reduced significance level, α_i, such that $\Sigma \alpha_i = \alpha$ (see Wright 1992). For taxa that were only abundant at two of the three habitats, individual t-tests using separate variances were performed. These taxa included *A. rupestris*, *D.* cepedianum, Amphipoda, Coleoptera, Diptera (excluding Chironomidae), Ephemeroptera, Isopoda, and Oligochaeta.

Results

Micropterus punctulatus samples pooled both temporally and spatially (for epigean, spring, and cave habitat-types) demonstrated trophic enrichment of nearly 2 δ^{13}C values and 2 δ^{15}N values (nearly one trophic level) for epigean versus both cave and spring samples (δ^{13}C: $F_{2,58}$: 9.408; P< 0.001 and δ^{15}N: $F_{2,58}$: 20.162; P< 0.001) (Figure 1).

Differences were between enriched $\delta^{13}C$ values for spring versus epigean habitat (df: 58;

P< 0.001) and enriched $\delta^{15}N$ values in epigean versus both cave (df: 58; P: 0.007) and

spring (df: 58; P< 0.001) habitats (Figure 1). T-tests were conducted for two of the

system's other top consumers, *L. macrochirus* and *L. megalotis*, because no samples were

found at cave sites. *L. macrochirus* in epigean habitat was enriched in $\delta^{15}N$ (t: 4.427; df:

28.2; P< 0.001) but not $\delta^{13}C$ (t: 0.038; df: 19.1; P: 0.970). This trend did not hold for *L.*

megalotis, however, as there was no significant difference between the habitats for either

$\delta^{13}C$ (t: 0.529; df: 68.3; P: 0.599) or $\delta^{15}N$ (t: 1.241; df: 59.3; P: 0.219) values.

Mid-level fish consumers demonstrated mixed results. *Cottus carolinae*

demonstrated no differences among habitats for either $\delta^{13}C$ ($F_{2,19}$: 0.992; P: 0.389) or

$\delta^{15}N$ ($F_{2,19}$: 1.016; P: 0.381) values (Figure 2). *A. rupestris* individuals were collected at

both epigean (N: 13) and spring (N: 8) sites, with no difference found between habitats

for $\delta^{13}C$ (t: -0.842; df: 11.8; P: 0.417) but $\delta^{15}N$ values revealing elevated values in

epigean habitat (t: 3.605; df: 16.6; P: 0.002). *D. cepedianum* were only collected in the

cave (N: 5) and epigean (N: 15) sites and revealed a similar trend, with no difference

between $\delta^{13}C$ values (t: -1.212; df: 11.7; P: 0.249) and higher $\delta^{15}N$ values in the cave

habitat (t: 3.438; df: 18.0; P: 0.003).

Likewise, *D. cepedianum* samples at individual cave and epigean sites did not

differ in $\delta^{13}C$ ($F_{2,17}$: 2.703; P: 0.080) values but did differ in $\delta^{15}N$ values ($F_{2,17}$: 7.910; P:

0.002). Multiple comparisons with a Bonferroni correction revealed that samples from

G4 were depleted compared to ERP (df: 16; P: .002) and G1 (df: 16; P: .008) in $\delta^{15}N$

values (Figure 3). No significant differences existed in *Pimephales notatus* among

habitats for δ^{13}C values ($F_{2,9}$: 0.524; P: 0.609), but a significant difference existed among

habitats for δ^{15}N values ($F_{2,10}$: 10.987; P: 0.003), with cave values enriched compared to

both spring (df: 10; P: 0.009) and epigean (df: 10; P: 0.009) habitats (Figure 4).

Comparisons of the two troglobitic species (*Typhlichthys subterraneus* and *Chologaster*

agassizi) and three common accidentals (*P. notatus*, *D. cepedianum*, and *M. punctulatus*)

revealed differences in both δ^{13}C ($F_{3,17}$: 28.302; P<0.001) and δ^{15}N ($F_{3,17}$: 6.297; P:

0.004) values. Differences, however, were only between enriched *P. notatus* δ^{13}C values

compared to both cave fish, *T. subterraneus* (df: 15; P<0.001) and *C. agassizi* (df: 15;

P<0.001), and between depleted *M. punctulatus* δ^{15}N values compared to *T. subterraneus*

(df: 15; P: 0.002) and *P. notatus* (df: 15; P: 0.044) (Figure 5).

Results for invertebrates further enforced the cave-spring similarities. Two

species of crayfish, the epigean *Cambarus tenebrosus,* and the cave crayfish, *Orconectes*

pellucidus show that differences could be distinguished between cave and epigean

habitats at the 1° consumer level for both δ^{13}C ($F_{4,21}$: 10.804; P<0.001) and δ^{15}N ($F_{4,21}$:

8.944; P<0.001) values (Figure 6). Differences were in depleted δ^{13}C values in cave *O.*

pellucidus individuals versus *C. tenebrosus* from all other habitat types (df: 20; all

P<0.003) and enriched δ^{15}N values in cave *O. pellucidus* versus cave *C. tenebrosus*

individuals (df: 20; P<0.001). Neither *O. pellucidus* (found only at cave and spring sites)

nor *C. tenebrosus*, however, differed between sites for either δ^{13}C or δ^{15}N values (df: 20;

all P>0.053).

ANOVA results run on Chironomidae members indicated a significant difference

among habitats for δ^{13}C values ($F_{2,16}$: 15.128; P< 0.001), with Bonferroni-corrected

multiple comparisons revealing enriched values between epigean and cave (df: 16; P< 0.001) and epigean and spring (df: 16; P: 0.027) habitats. There were also statistical differences among habitats for $\delta^{15}N$ values ($F_{2,16}$: 6.354; P: 0.009), with enriched cave values versus both epigean (df: 16; P: 0.034) and spring (df: 16; P: 0.016) habitats (Figure 7). All remaining invertebrate comparisons were between spring and epigean habitats, except for samples from the order Diptera (including all dipterans except chironomids), which were between cave and epigean habitats (Table 4). Among all comparisions, only results for Coleoptera and Diptera revealed significant differences, with epigrean Colepterans enriched in $\delta^{13}C$ compared to spring individuals (t: 2.538; df: 6.0; P: 0.044) and cave Dipterans (excluding chironomids) enriched in $\delta^{15}N$ compared to epigean individuals (t: 5.879; df: 4.0; P: 0.004).

At the base of the food web in the three systems, tests were done to compare bacterial and detrital samples; algal samples were abundant only in epigean sites and so comparisons were not made to other habitats. A t-test between bacterial samples (N: 8) from spring and epigean sites revealed no significant differences for either $\delta^{13}C$ (t: 0.982; df: 3.2; P: 0.395) or $\delta^{15}N$ (t: 0.036; df: 4.2; P: 0.973) values. There were no significant differences among detrital samples from the three habitats for $\delta^{13}C$ ($F_{2,10}$: 0.636; P: 0.549) or $\delta^{15}N$ ($F_{2,17}$: 2.305; P: 0.130) (Figure 8).

Discussion

Among Habitat Comparisons: Despite the variation existing for *M. punctulatus* both temporally and spatially, pooled samples revealed a clear trend in contrast to the

hypothesis that the spring sites would display intermediate values for both $\delta^{13}C$ and $\delta^{15}N$ to values from cave and epigean sites. Rather, spring sites function similarly to cave sites, which both were shown to be detritus-driven systems. This contrasts the epigean food web, which is more complex and most likely presents a case of multiple basal nutrient inputs. Though this evidence agrees with River Continuum Concept predictions of multiple nutrient inputs for mid-reach streams (orders 4-6) (Vannote et al. 1980), what is surprising is the evidence that *M. punctulatus* specimens found in the spring heads are remaining in the springs to feed despite their access to what would appear to be an excess of food sources in the hydrologically connected main-stem. This phenomenon may be explained in part by the ephemeral nature of the hydrological connection between a particular spring site and the main-stem. That is, during low-flows, many of the associated tributaries (ranging from less than 3 m for PS and SC to greater than 25 m for ES and RS) connecting a given spring to the main-stem were either extremely shallow or (as was often the case in SC) even ephemeral. However, these periods were sporadic and, given the month-long assimilation period for $\delta^{13}C$ and $\delta^{15}N$ in high-end consumers, this phenomenon appears to be more an artifact of behavior than of geographical isolation.

The notion that higher-order consumers were more affected by differences in habitat was confirmed by the lack of difference found in mid-level consumers, specifically for *C. carolinae* among habitats (Figure 2) and *D. cepedianum* between cave and epigean habitats (Figure 3). Though there was a significant difference between G2 and all other sites (G1, G4 and pooled cave sites), pooling all epigean data results in the same trend: cave and epigean sites show no difference in either $\delta^{13}C$ or $\delta^{15}N$. This, in

addition to supplemental evidence provided by gut-contents analysis for *D. cepedianum*

and other accidental species, suggests that most accidentals are not able to assimilate cave

nutrients and, consequently, are starving soon after they happen into the cave. Anecdotal

evidence for this was also observed by the physically degraded state of the larger fish

physically observed in the cave: they were slow-moving, pale, and had likely begun to

metabolize muscle tissue for energy. Two of these larger accidentals were *Pomoxis*

annularis, one from DS (TL: 200 mm; δ^{13}C: -23.27; δ^{15}N: 13.74) and another from ERP

(TL: 183 mm; δ^{13}C: -26.03; δ^{15}N: 14.19); additionally, one *Cyprinus carpio* (TL: 760

mm; δ^{13}C: -24.04 δ^{15}N: 11.79) was found in the cave, at site DS. Both of the *P.*

annularis individuals displayed enriched δ^{15}N values compared to mean values for

spring-captured individuals (mean δ^{15}N: 13.36), which would support the starvation

theory, since processing of an individual's own tissue would lead to trophic enrichment of

δ^{15}N.

Results for *P. notatus* seem to contradict the trends of similarity between the cave

and spring trophic structures, with δ^{15}N values from fish in spring and epigean habitats

being lower than for individuals from the cave (Figure 4). This, however, may be a relict

of the size class of individuals between sites, as specimens from the cave were all young

of the year fish (mean TL: 39.6 mm) that had most likely derived most of their biomass

from organic matter in their yolk sacs. This may also explain the unexpected placement

of *P. notatus* at a higher level (higher δ^{15}N values) on the cave food web than at other

habitats. The isotope signatures of larval fish should be similar to those of a predator of

the parental species, since they are metabolizing organic matter derived directly from the

main-stem adult, and, in theory, their $\delta^{15}N$ signal should be one trophic level higher then their parent. Over time, as the larvae shifts to exogenous food sources, the isotopic signature would shift to reflect the individual's planktivorous feeding habits. This is an intriguing idea that deserves further study.

In contrast to the fish, crayfish isotopes did not differ among habitats. The distribution of isotope values for eyeless crayfish (*O. pellucidus*) was very tight but not different between habitats (Figure 6). The only statistical differences found were between species, which is nonetheless interesting based on the similar life-history and feeding strategies between the two species. This is especially surprising for the epigean species found in the cave sites, which had no access to surface nutrient inputs. However, this may be explained by the distribution of species among cave sites: *C. tenebrosus* samples were found only in OC, whereas *O. pellucidus* samples were taken from RSS and ERP. This is significant because of the relatively high flows witnessed in OC, often with visible anthropogenic waste coming in from undisclosed sources. There are currently fourteen identified surface drainages (sinkholes, sinking streams, etc.) that drain to OC (Joe Mieman, Hydrologist, MCNP, personal communication), which means OC may be highly influenced by surface streams. Consequently, despite the fact that OC is separated from its intermediary spring (SC) by a large hydrological distance, *C. tenebrosus* individuals taken from OC may only have been in the cave environment for a short time due to high flows and may not have had time to incorporate the cave isotopic signature.

Results for chironomids further support the hypothesis that cave and spring sites share similar basal nutrient inputs, with differences in $\delta^{13}C$ values between epigean and

all other habitat types (cave and spring) (Figure 7). ANOVA results for δ^{15}N values, however, demonstrated differences between cave and all other habitat types (epigean and spring), with cave values actually elevated above the other two habitat types, a result not be expected based on *a priori* hypotheses. Unlike the elevated δ^{15}N cave values for *P. notatus*, which may be explained as an artifact of reliance on endogenous feeding on the yolk sac, it remains unclear why δ^{15}N values were significantly elevated for chironomids within the cave environment, but starvation remains a possible explanation.

At the basal level, detrital inputs displayed no differences between habitat types for cave, spring and epigean site-types (Figure 8). This seems to suggest that organisms lower on the trophic food web display less variation, which confirms the understanding that variation among food web components is more pronounced as energy moves up the food web. Indeed, in our system, with the cave and spring habitats containing fewer organisms in the food web, fewer discrete trophic levels were expected as compared to the food web of the epigean habitat. Additionally, the consistency of the signal (non-significant δ^{13}C values for detritus across habitats) enhances our faith that the utility of the detrital signal as a basal gauge for comparing our various habitat types is robust.

Examination of composite graphs, pooled temporally and spatially (among sites), from the three systems reveals that the epigean food web generally displayed a wider range (δ^{13}C values from –23 to –30), indicating probable input from a mixture of algal and detrital signals (Figure 9). In contrast, spring and cave systems encompassed a narrower range (spring: -24 to –29 δ^{13}C values; cave: -21.5 to –27.5) when the influence of extraneous values (such as algae and the terrestrial signal, moss, for spring samples

and an accidental tadpole, for the cave) were removed (Figures 10, 11). The cave food web is even further compressed with the elimination of all accidentals and the ostracod signal (-22.5 to -26.5). Additionally, in both spring and cave food webs, all signals (excluding cave accidentals and ostracods) fall slightly to the right of the detrital signal, nestled completely between the detrital and bacterial signals. Simon et al. (2003) found that bacteria have a much more pronounced affect on cave food webs than originally expected. This seems to hold for not only our cave system, but also our spring system, giving further evidence of the similarities between these two systems. However, due to the nature of the bacterial sample collections, these values represent a hybrid of all signatures found in the first 10 mm of benthic sediments.

As expected, the height (δ^{15}N values) of the epigean trophic structure is more pronounced than that of the cave, which has a less complex food web, though the difference was not as pronounced as might have been expected (only 1-2 δ^{15}N values between top-end predators, with detrital signals almost identical). Interestingly, the spring system shows this same trend of higher top-level consumers, with δ^{15}N values slightly higher than in the cave system, though this was noted primarily in *Micropterus salmoides*, a voracious and highly motile predator, which may have been utilizing the epigean habitat more than other fish. The reason for the noticibly (but not statistically significant) lower detrital signal (2 δ^{15}N values) in the spring versus the other two systems remains unclear.

That the vertical axis of the epigean food web is compressed compared to the cave system is underscored by accidentals found in the cave system. Though the two *P.*

annularis individuals showed δ^{15}N signals close to the mean *T. subterraneus* signal, the

values of such mid-level consumers such as *P. notatus* and *D. cepedianum* were elevated

relative to the cave trophic structure (but not compared to other values of similar fish in

the other food webs), suggesting that these organisms were recent accidentals from

outside systems and/or that they were not incorporating the cave signatures and were, in

essence, starving, the latter of which would be consistent with visual observations from

the accidental captures. That miscellaneous epigean larval fish exhibited the highest cave

signature (a 2 δ^{15}N value increase from the top-end cave predator) underscores the notion

of elevated epigean and spring vertical trophic structure, as the isotopic signatures of

these fish were most likely the result of the composition of epigean nutrients in their yolk

sacs.

Among Site Comparisons: The major causes of variation among sampling sites

within each habitat type remains unclear. One suspicion was that different hydrologic

characteristics at the different areas could have been caused by an impoundment (Lock

and Dam 6) placed just outside the western border of MCNP by the Army Corps of

Engineers nearly 100 years ago, potentially causing trophic differences along the artificial

gradient from the impounded (G1) to free flowing (G4) sections of the Green River. This

suspicion remains in question, however, as only G2 deviated from the other sites,

suggesting some other mechanism may have caused this difference.

It is difficult to determine the effects the impoundment may have had on other

trophic patterns, but our data suggest that larger fish species may have been relatively

unaffected trophically. Differences between spring sites were even less pronounced, suggesting that these habitats are both trophically independent of their main-stem hydrologic connection to the Green River and trophically similar despite their observed discrepancies in species composition. This latter point can be seen by examining the distribution of *Lepomis sp.* found in the various spring sites. More *L. megalotis* were found at the upstream springs (e.g., PS, RS and ES) and more *L. macrochirus* at Sand Cave spring. *Lepomis megalotis* is typically more abundant in flowing waters whereas *L. macrochirus* is more abundant in low-gradient streams and impoundments (Pflieger 1997). Despite these inherent differences in species composition, few differences were found in the overall trophic structure of these or other species between given spring sites.

Differences seen between sites for *M. punctulatus* reflect the variation seen for the same species for temporal comparisons. That most of the variation is occurring within comparisons of the highest order consumer for this system suggests that high-order consumers tend to be less stable within the local food web. Though this variation may be a relict of additive shifts up the food chain caused by slight basal variation, it may also reflect the more opportunistic feeding strategies of *M. punctulatus* and other high-order consumers, both seasonally (year-to-year) and spatially (site-to-site). One major implication of these observations is that the local systems studied are driven primarily by bottom-up temporal and spatial pressures.

Temporal Comparisons: The lack of evidence for temporal differences between sites suggests that year to year variation may be minimal in trophic position and basal

nutrient derivation for the upper-level consumers tested. Of the variation present between

sampling years, most occurred in $\delta^{13}C$, with values for *M. punctulatus* contributing the

most variation evidenced through statistically different t-tests. This suggests that while

trophic position appears to remain fairly constant over time, basal nutrient inputs may

shift. This occurrence would seem to be the result of shifts in nutrient uptake at the

bottom of the food web rather than a complete shift in feeding strategy for the upper-level

consumer. It is interesting to note that in all cases for *M. punctulatus*, the top predator in

our study, all tests for differences in $\delta^{13}C$ were significant, while none for $\delta^{15}N$ revealed

significant differences. In this case, fluctuations in basal detrital inputs may have been

enough to reveal significances between years, while $\delta^{15}N$ values, which normally exhibit

more variation up the food web, are highly conserved, indicating both a consistent food-

web structure as well as feeding strategy among these individuals.

Conclusion

Of the two *a priori* hypotheses established at the outset of this experiment, only

the hypothesis that fish and invertebrates living in spring heads should express $\delta^{13}C$

values intermediate to those of organisms in cave and epigean aquatic systems was

refuted. Though cave and spring systems were dominated by allochthonous leaf litter

characteristic of headwater streams (orders 1-3), the epigean system also indicated a large

dominance in detrital inputs. Primary differences in $\delta^{13}C$ were seen instead at higher

trophic levels, particularly in top consumers (*i.e.*, *Lepomis* species), where $\delta^{13}C$ values

decreased from epigean to spring to cave habitats. Additionally, the data suggested that

bacteria may be an important nutrient source for the cave food web.

Overall, trophic compression could be seen in cave and spring compared to epigean habitats; however, despite relatively compressed trophic levels of cave and spring habitats, δ^{15}N values of accidental species tended to be enriched, even when compared to epigean signals. This was attributed to one of two effects: trophic enrichment from yolk sacs (with the parent as the effective "prey"), or starvation, which leads to self-processing and trophic enrichment through differential metabolism of light isotope. These results suggest that most accidentals that are swept into Mammoth Cave are not thriving and, instead, starve after a short time. Though this does not negate the possible threat of stocked game species (e.g., *Oncorhynchus mykiss* or *Esox masquinongy*) to indigenous cave fauna, the cave may act as a natural barrier preventing threats from such species. Ultimately, the role of flooding events that back water into the cave needs to be examined. These events are periodic and often substantial, providing interesting scenarios for examining nutrient pulses and the subsequent fate of nutrients (including fish) after they are swept into the cave. Determining whether these punctuated events provide detectable nutrient pulses that can be monitored via stable isotope analysis will be an important component of future subterranean studies.

Table 1. List of site descriptions and site designator codes. Latitude and longitude

coordinates could not be determined in cave sites because of the inability of GPS to work

underground.

Site	Type	Site Code	Lat.	Long.
Dead Sea	Cave	DS		
Echo River Proper	Cave	ERP		
Owl Cave	Cave	OC		
River Styx Shallow	Cave	RSS		
Echo Spring	Spring	ES	37.17953	86.11236
Pike Spring	Spring	PS	37.21457	86.05535
River Styx Spring	Spring	RS	37.19085	86.10733
Sand Cave Spring	Spring	SC	37.17008	86.14836
Green River 1	Epigean	G1	37.21539	86.25116
Green River 2	Epigean	G2	37.17035	86.16175
Green River 3	Epigean	G3	37.20092	86.10909
Green River 4	Epigean	G4	37.21481	86.05001

Table 2. Taxa, symbols and gross sample numbers used in computing statistics. Samples for fish species corresponded to one individual per sample. Each invertebrate sample ranged from N =1 to N = 80 individuals.

Taxa	Symbol	Number of Samples		
		Cave	Spring	Epigean
algae	A		5	1
Ambloplites rupestris	Ar		8	13
Amphipoda	Am		5	4
Aplodinutis grunniens	Ag			3
bacteria	B	1	4	4
Cambarus tenebrous	Ct	6	4	4
Camberidae	Cm			3
Campostoma oligolepis	Co		1	
Chironimidae	C	7	6	6
Chologaster agassizi	Ca	4		
Coleoptera	Col		3	5
Cottus carolinae	Cc	2	10	6
Cyprinella sp.	Cy		1	
Cyprinus carpio	Cca	1		
detritus	D	6	7	7
Diptera	Di	6	1	3
Dorosoma cepedianum	Dc	5	2	15
Ephemeroptera	E		4	5
Etheostoma nigrum	En		1	
fungal mycelia	Fm	2		
Hemiptera	H			4
Ictiobus bubalus	Ib			2
Isopoda	I	1	4	4
juvenile fish	J	1		
Labidesthes sicculus	Ls		1	
larval fish	L	1		
Lepisosteus osseus	Lo			3
Lepomis cyanellus	Lc		9	
L. macrochirus	Lm		31	6
L. megalotis	Lme		37	46
Micropterus punctulatus	Mp	4	29	28
M. salmoides	Ms		2	5
Minytrema melanops	Mm		1	
moss	M		9	3
Neroptera	N		1	2
Odonata	Od		1	5
Oligochaeta	O	1	4	4
Orconectes pellucidis	Op	8	3	
Ostracoda	Os	1		
Pimephales notatus	Pn	6	4	4

Table 2, continued.

Plecoptera	P			2	4
Polymontiadae	Pol	1		4	2
Pomoxis annularis	Pa	2		2	
Pylodictis olivaris	Po				4
Semotilus atromaculatus	Sa			2	
tadpole	T	2			
Tricoptera	Tr				2
Typhlichthys subterraneus	Ts	5			
zooplankton	Zo	1			
Zygoptera	Z			1	5

Table 3. Results of twenty-one unpaired t-tests comparing δ^{13}C and δ^{15}N values from the 2002 and 2003 sampling years. Bolded values indicate significance using an adjusted P-value of 0.0027 for multiple comparisons. Results are reported from tests of unequal variance for each group. All tests were for a given species except where grouped by genus (*) when insufficient samples were available.

Site	Species	δ^{13}C				δ^{15}N			
		N	t-value	df	P	N	t-value	df	P
G1	*Lepomis megalotis*	13	-1.806	10.2	0.1	13	1.165	10.9	0.269
G2	*L. megalotis*	12	8.587	8.2	**0**	12	0.285	6.9	0.784
G3	*L. megalotis*	10	-0.898	5.1	0.41	10	0.647	6.3	0.54
G3	*Micropterus punctulatus*	10	2.919	7.8	0.02	10	1.579	7.8	0.154
G4	*L. megalotis*	10	-5.097	4.4	0.005	10	1.746	4.7	0.145
G4	*M. punctulatus*	8	-0.569	6	0.59	8	2.37	6	0.056
RS	*Lepomis sp.**	14	0.051	2.1	0.964				
RS	*L. megalotis*					9	0.415	5.9	0.693
RS	*L. macrochirus*					10	3.09	6.5	0.019
ES	*Lepomis sp.**	15	1.668	5.1	0.155	15	3.854	7.6	0.005
SC	*L. megalotis*	10	0.537	6.3	0.61	10	-0.09	8	0.931
PS	*M. punctulatus*	11	4.377	8.3	**0.002**	11	2.171	6.1	0.072

Table 4. Spatial comparisons of δ^{13}C and δ^{15}N values within habitat types among respective cave (C), spring (S), and epigean (E) sites. T-values represent cases where taxa were collected only at two sites for a given taxa; in all other cases, ANOVA tests were performed. Bolded values represent significance. Multiple comparisons for *Micropterus punctulatus* within the spring habitat (1) reveled that ES was trophically enriched compared to both PS (df: 25; P: 0.003) and SC (df: 25; P: 0.013) in δ^{13}C. Multiple comparisons for *Dorosoma cepedianum* (2) revealed δ^{15}N enrichment only between G1 and G4 (df: 12; P: 0.014). Despite a significant difference in *Lepomis megalotis* (3) among epigean habitat sites, multiple comparisons failed to reveal any differences among sites. Multiple comparisons for *M. punctulatus* (4) revealed an enriched δ^{13}C signal for site G1 compared to all other sites (df: 24; all P $\leq$ 0.001) and an enriched δ^{15}N signal in G3 compared to G4 (df: 24; P: 0.016).

Habitat	Taxa	δ^{13}C				δ^{15}N			
		t-value	F-ratio	df	P	t-value	F-ratio	df	P
C	*Orconectes pellucidus*	-1.527		5.7	0.18	0.829		2.6	0.476
S	*Lepomis macrochirus*		1.526	3	0.234		0.421	3	0.74
S	*L. megalotis*		0.62	3	0.608		2.395	3	0.086
S	*Micropterus punctulatus* [1]		5.855	3	**0.004**		2.934	3	0.053
E	*Ambloplites rupestris*	-1.943		5.1	0.108	3.659		4.9	0.015
E	*Dorosoma cepedianum* [2]		2.947	2	0.091		6.241	2	0.014
E	*L. megalotis* [3]		3.202	3	0.033		1.563	3	0.213
E	*M. punctulatus* [4]		11.807	3	**0**		4.953	3	0.008

Table 5. Summarized t-test data for various invertebrate taxa. Bolded values represent significance. All comparisons were between epigean and spring habitats except for members of the family Diptera (*), which were between epigean and cave habitats.

Taxa	$\delta^{13}C$				$\delta^{15}N$			
	N	t-value	df	P	N	t-value	df	P
Amphipoda	9	2.12	5.7	0.081	9	-0.18	6.9	0.862
Coleoptera	8	2.538	6	**0.044**	8	0.684	5.9	0.52
Diptera*	9	-0.311	2.4	0.781	9	5.879	4	**0.004**
Ephemeroptera	9	0.814	3.3	0.47	9	-0.73	4.1	0.505
Isopoda	8	-0.909	4.3	0.411	8	-1.348	4.6	0.24
Oligochaeta	7	-0.507	3	0.647	8	-1.442	5.2	0.207
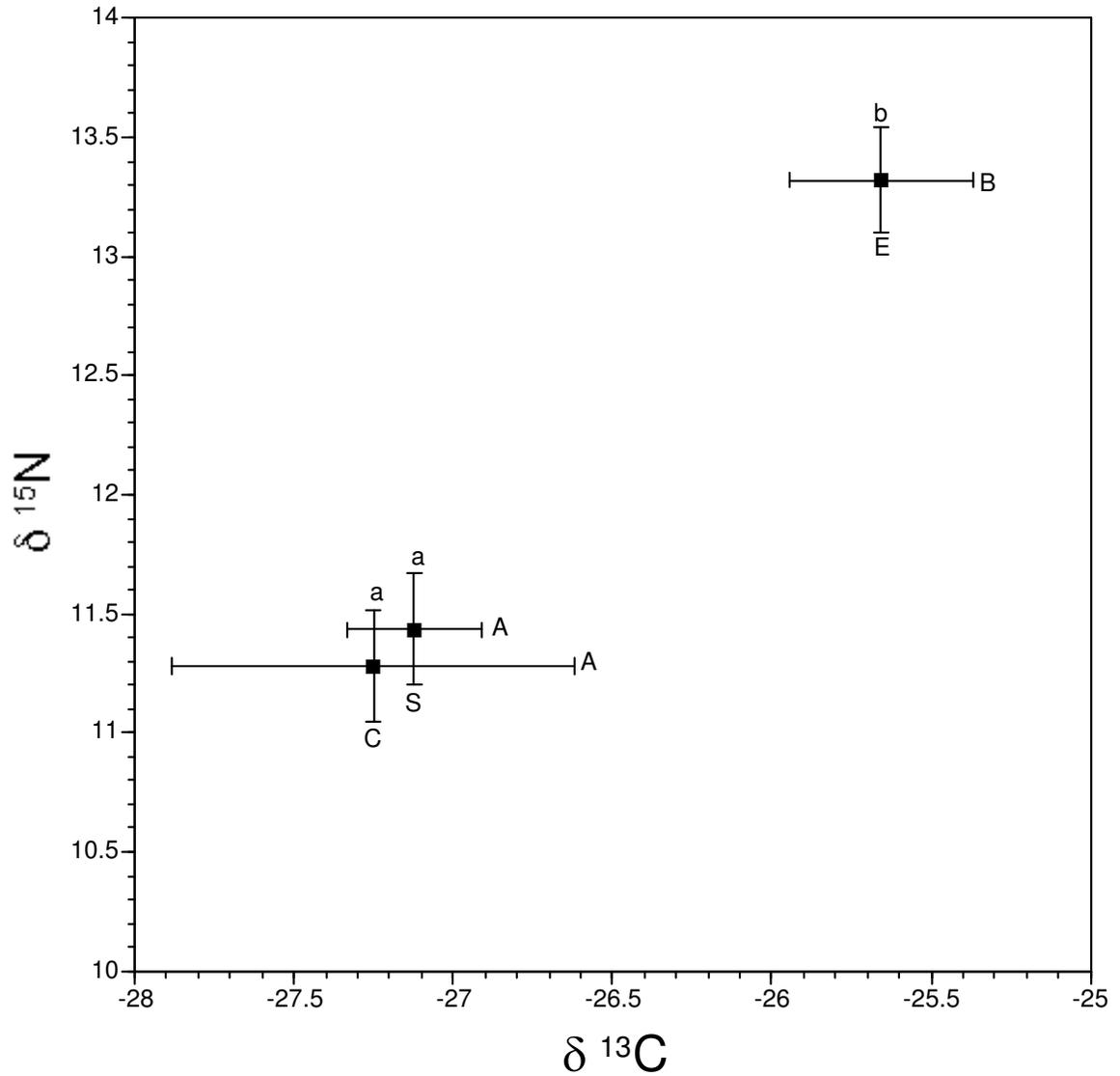
Figure 1. Temporally and spatially pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of *Micropterus punctulatus* for cave (CA), spring (SP) and epigean (EP) sites. Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.
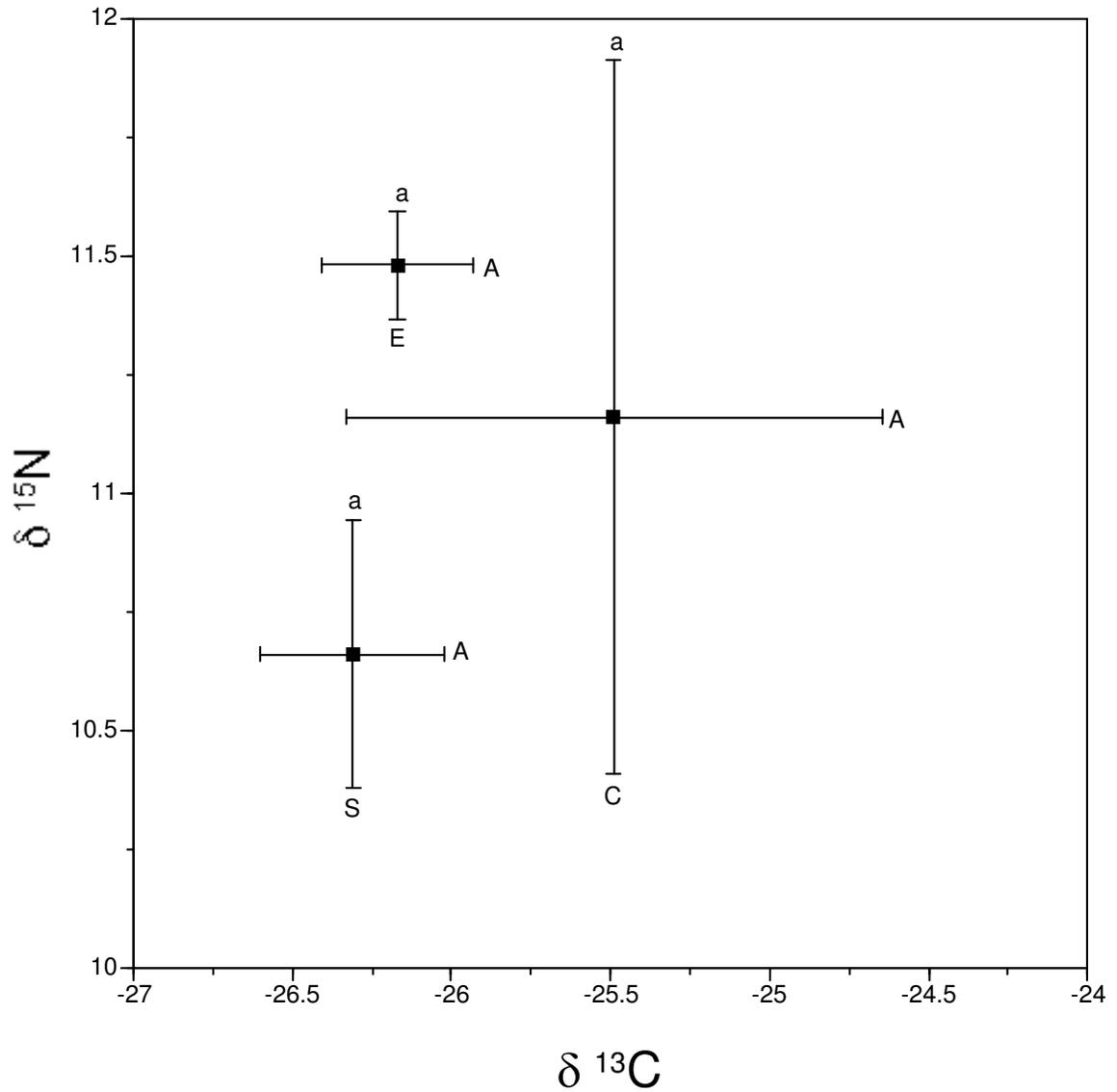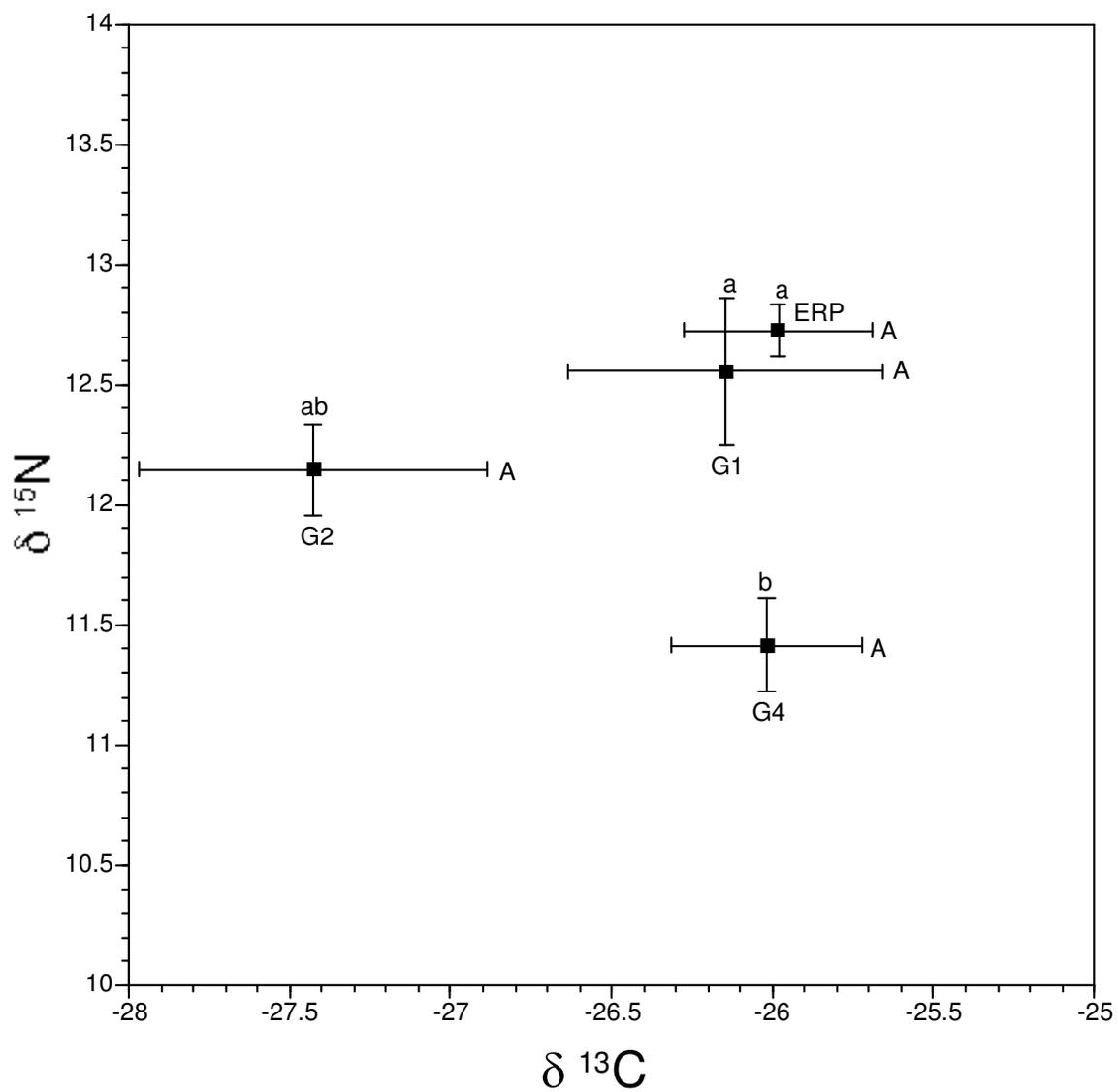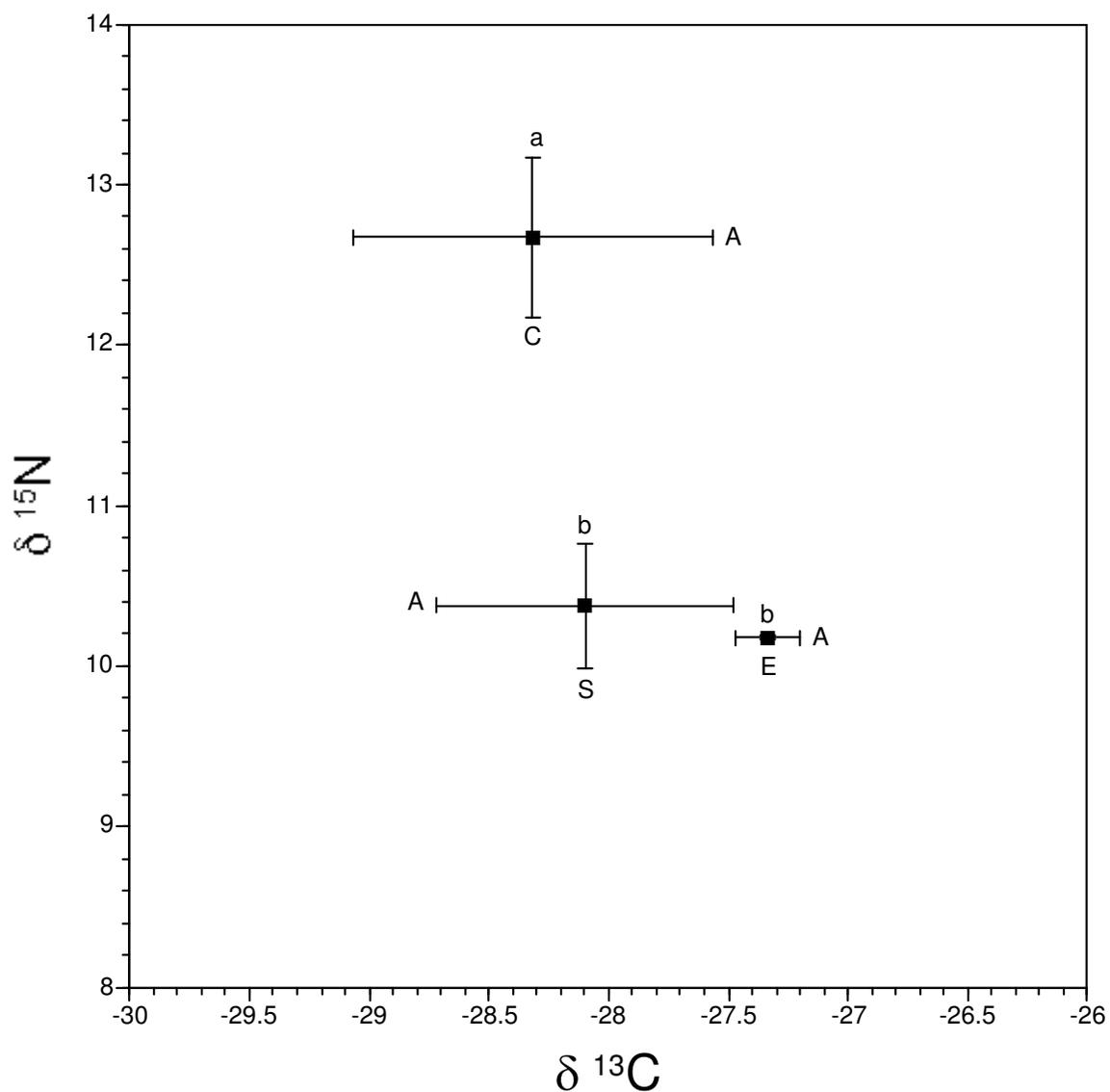
Figure 2. Temporally and spatially pooled mean (±1 SE) δ^{13}C and δ^{15}N values of *Cottus carolinae* for cave (C), spring (S), and epigean (E) sites. Differing lower-case letters designate significant differences in δ^{15}N values and differing upper-case letters designate significant differences in δ^{13}C values.

Figure 3. Temporally pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of *Dorosoma cepedianum* for main-stem sites (G1, G2, and G4) and a representative cave site (ERP). Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.

Figure 4. Temporally and spatially pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of *Pimephales notatus* for cave (C), spring (S), and epigean (E) sites. Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.
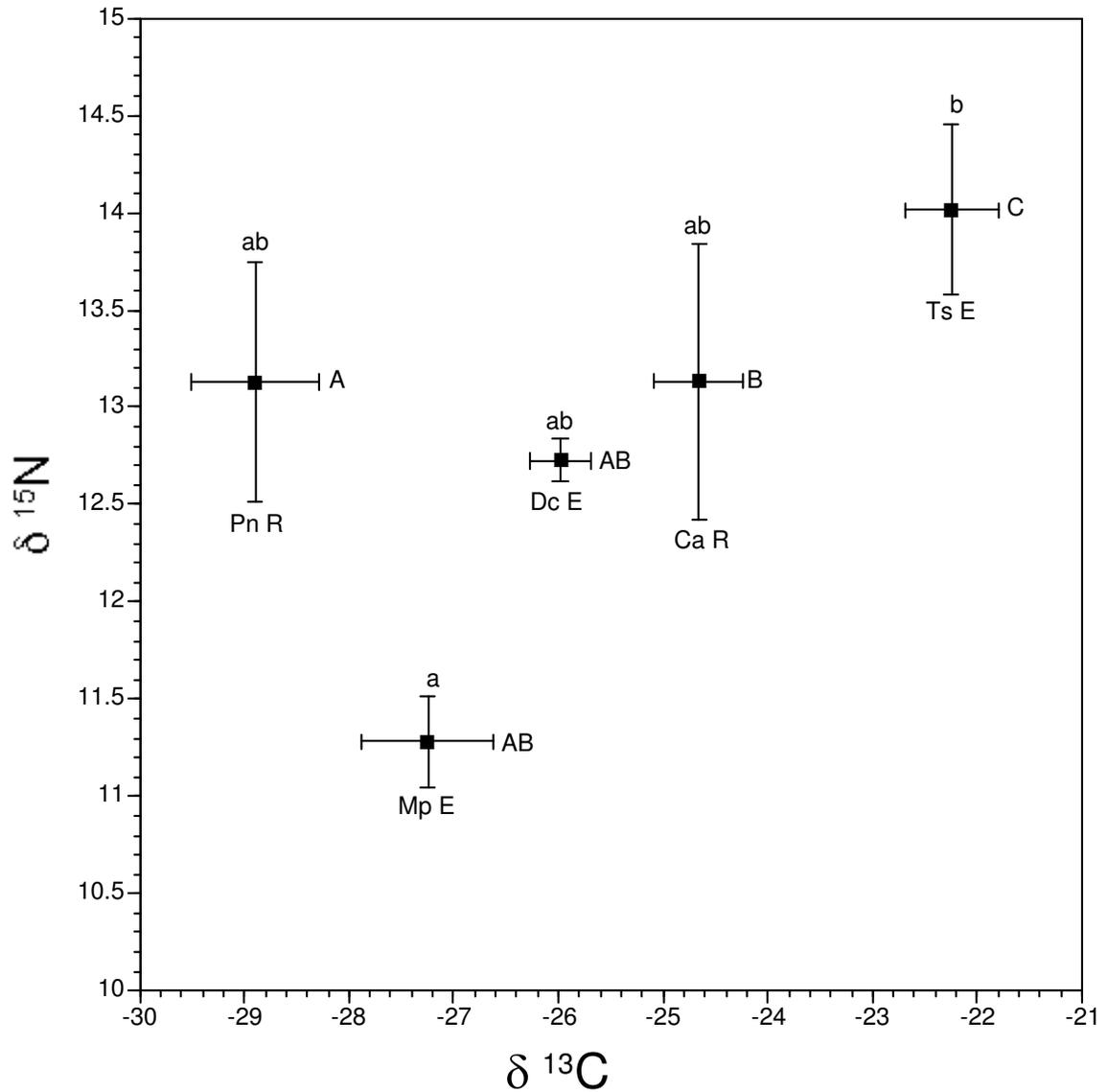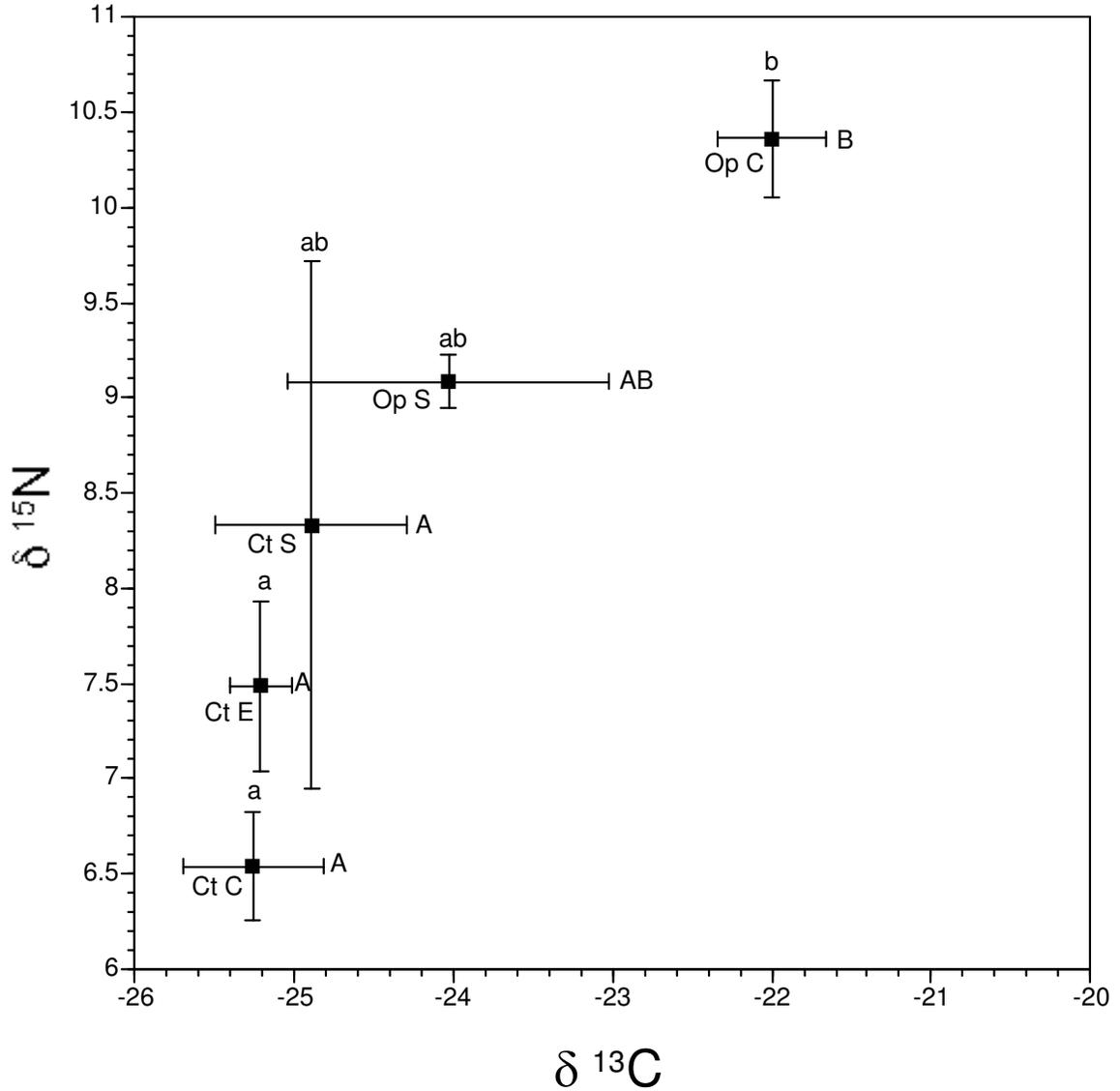
Figure 5. Mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of three accidental epigean fish, *Pimephales notatus* (Pn), *Dorosoma cepedianum* (Dc) and *Micropterus punctulatus* (Mp) and two cave fish, *Chologaster agassizi* (Ca) and *Typhlichthys subterraneus* (Ts) for two cave sites, River Styx Shallow (R) and Echo River Proper (E). Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.
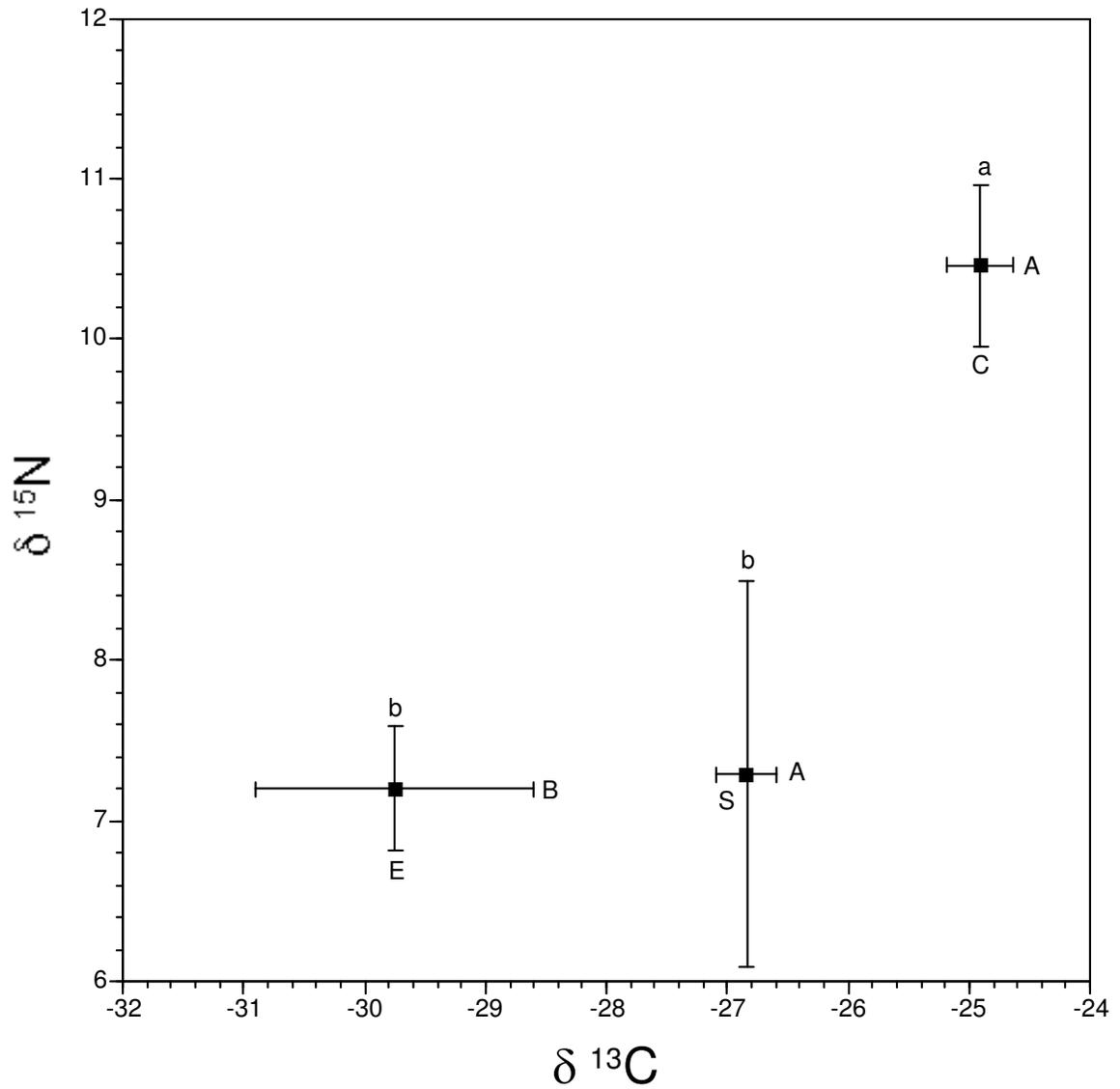
Figure 6. Temporally and spatially pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of two species of crayfish, the epigean crayfish, *Cambarus tenebrosus* (Ct) and the cave crayfish, *Orconectes pellucidus* (Op) for cave (C), spring (S), and epigean (E) sites. Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.

Figure 7. Temporally and spatially pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of member of the family Chironomidae for cave (C), spring (S), and epigean (E) sites. Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.

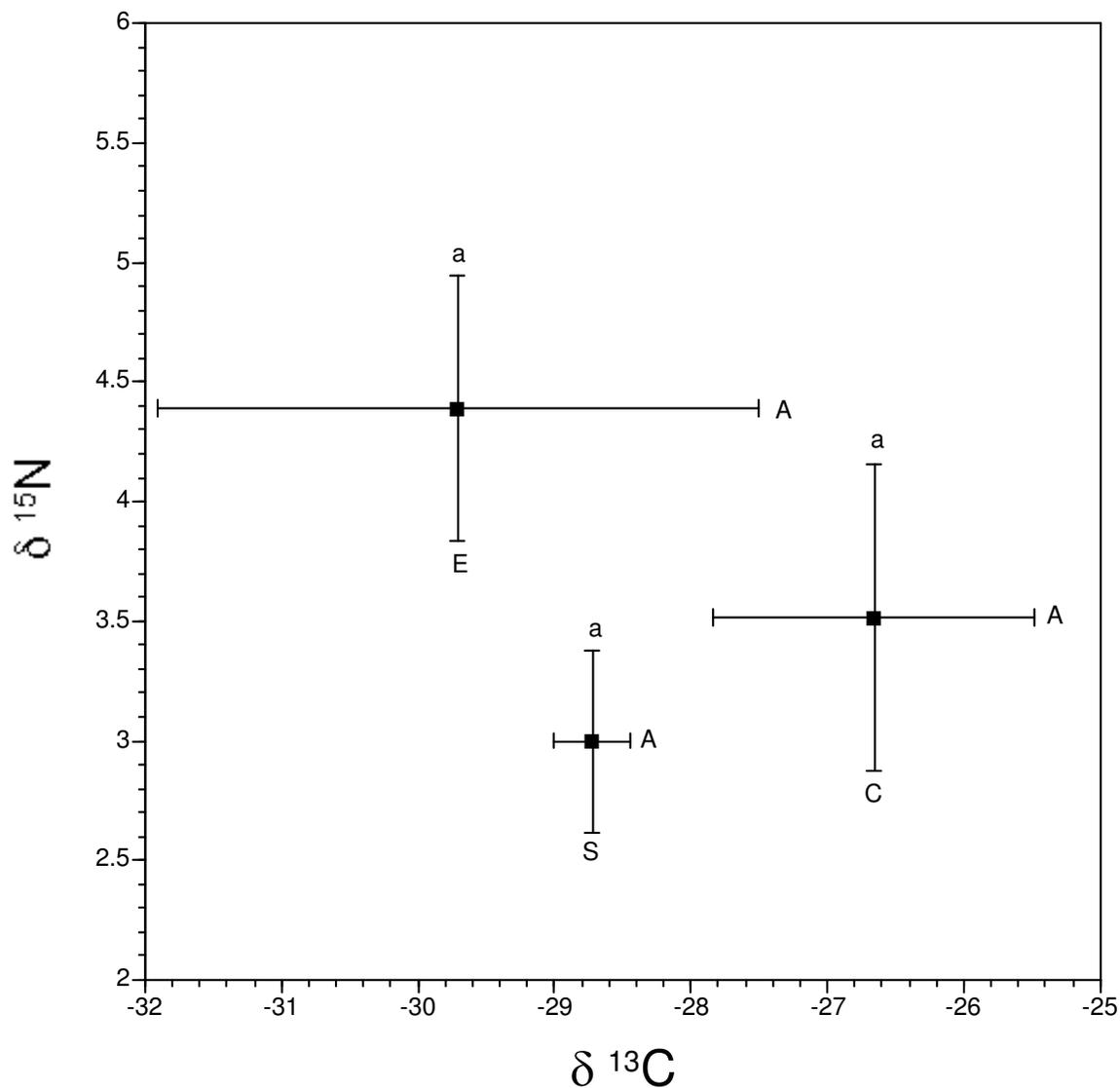Figure 8. Temporally and spatially pooled mean (±1 SE) $\delta^{13}C$ and $\delta^{15}N$ values of detritus samples for cave (C), spring (S), and epigean (E) sites. Differing lower-case letters designate significant differences in $\delta^{15}N$ values and differing upper-case letters designate significant differences in $\delta^{13}C$ values.
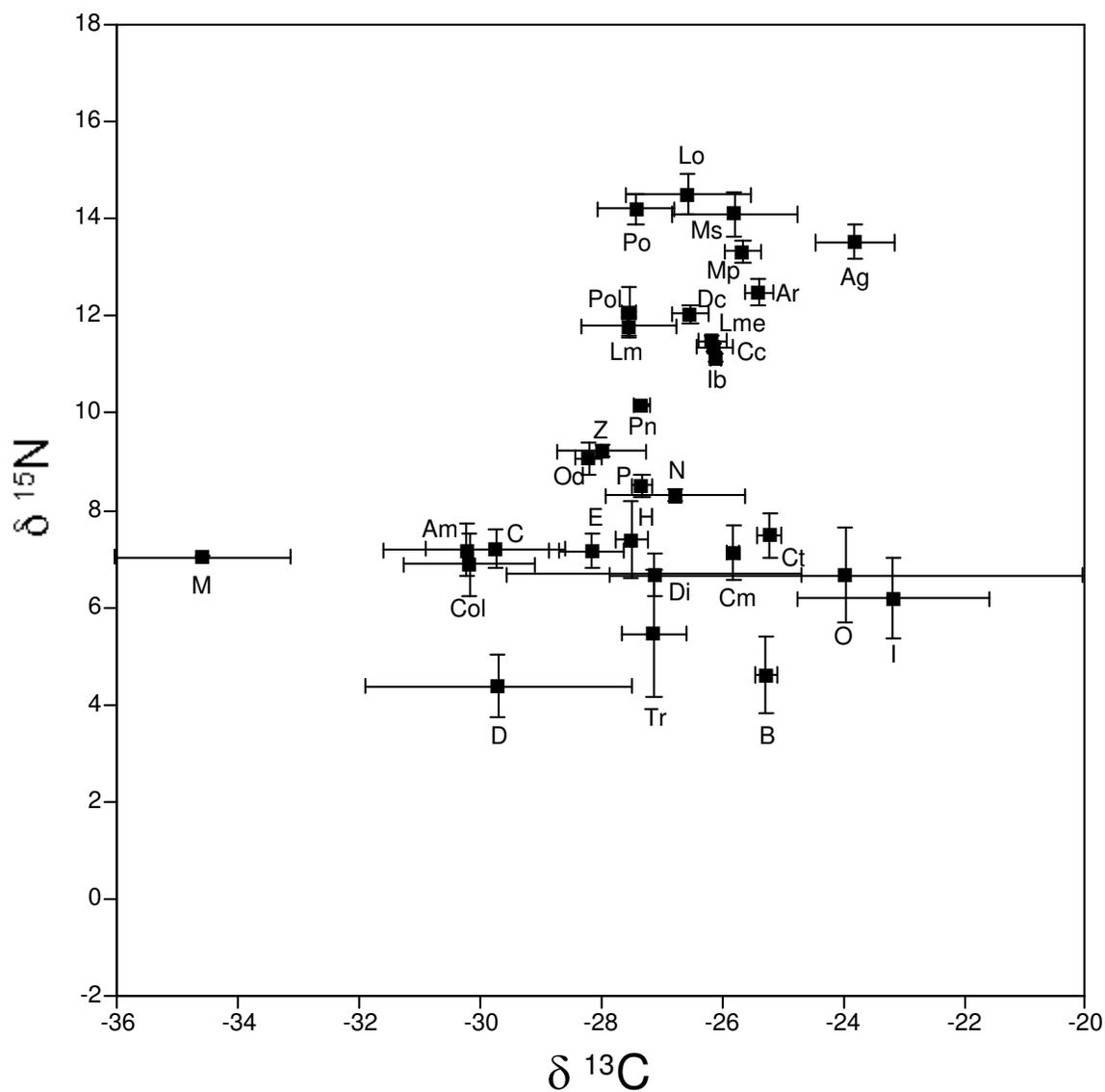
Figure 9. Epigean composite graph of temporally and spatially (among site) pooled δ^{13}C and δ^{15}N data, expressed with the following symbology: *Ambloplites rupestris* (Ar); Amphipoda (Am); *Aplodinotus grunniens* (Ag); bacteria (B); Camberidae (Cm); Chironimidae (C); Coleoptera (Co); *Cottus carolinae* (Cc); detritus (D); Diptera (Di); *Dorosoma cepedianum* (Dc); Ephemeroptera (E); *Orconectes pellucidis* (Op); Hemiptera (H); *Ictiobus bubalus* (Ib); Isopoda (I); *Lepomis macrochirus* (Lm); *L. megalotis* (Lme); *Lepisosseus osseus* (Lo); *Micropterus punctulatus* (Mp); *M. salmoides* (Ms); moss (M); Neroptera (N); Odonata (Od); Oligochaeta (O); *Pimephales notatus* (Pn); Polymontiadae (Pol); *Pylodictis olivaris* (Po); Plecoptera (P); Tricoptera (Tr); and Zygoptera (Z).
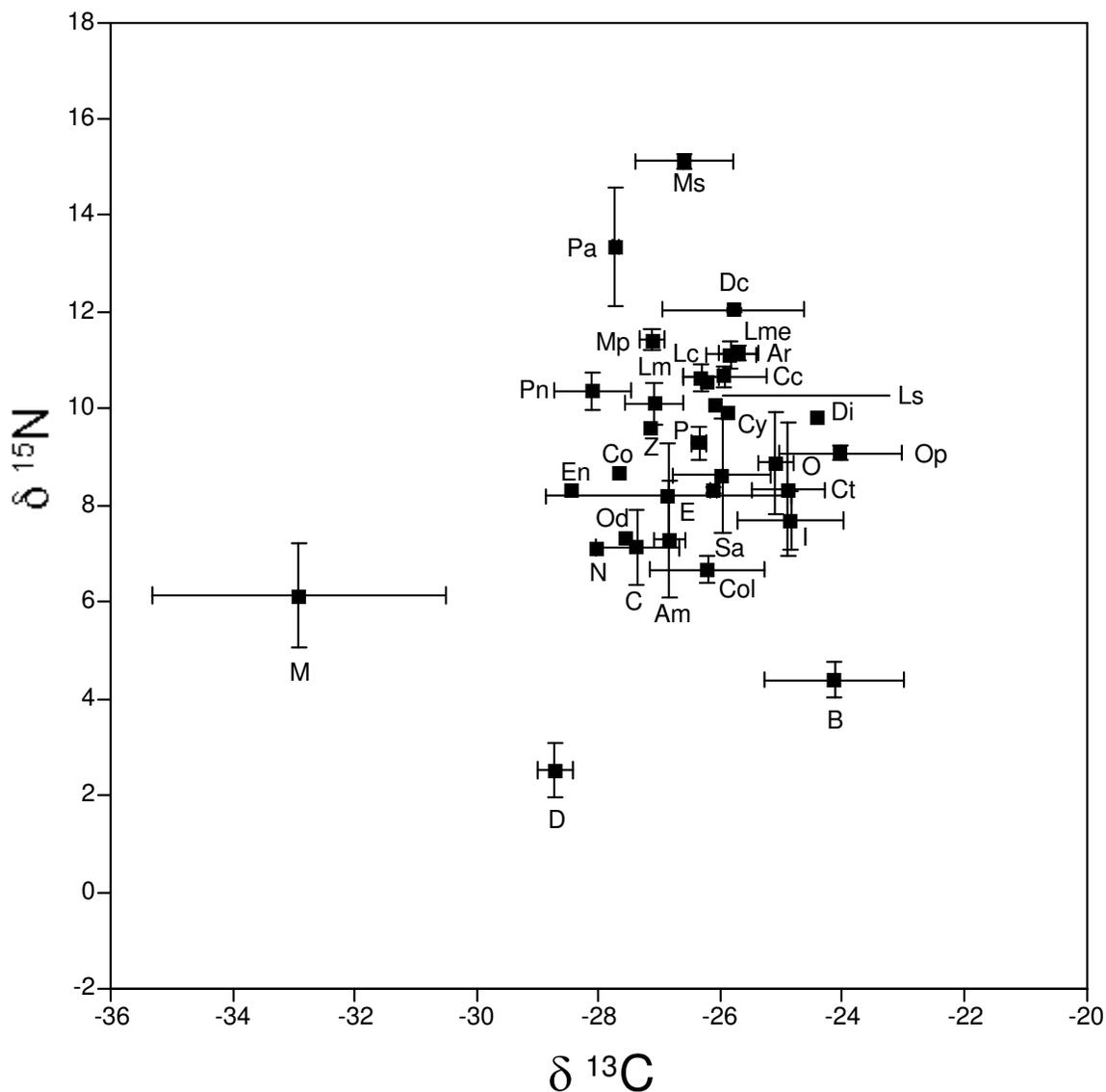
Figure 10. Spring composite graph of temporally and spatially (among site) pooled $\delta^{13}C$ and $\delta^{15}N$ data, expressed with the following symbology: *Ambloplites rupestris* (Ar); Amphipoda (Am); bacteria (B); *Camberus tenebrous* (Ct); *Campostoma oligolepis* (Co); Chironimidae (C); Coleoptera (Col); *Cottus carolinae* (Cc); *Cyprinella sp.* (Cy); detritus (D); Diptera (Di); *Dorosoma cepedianum* (Dc); Ephemeroptera (E); *Etheostoma nigrum* (En); Isopoda (I); *Labidesthes sicculus* (Ls); *Lepomis cyanellus* (Lc); *L. macrochirus* (Lm); *L. megalotis* (Lme); *Micropterus punctulatus* (Mp); *M. salmoides* (Ms); *Minytrema melanops* (Mm); moss (M); Neroptera (N); Odonata (Od); Oligochaeta (O); *Orconectes pellucidis* (Op); *Pomoxis annularis* (Pa); *Pimephales notatus* (Pn); Plecoptera (P); *Semotilus atromaculatus* (Sa); and Zygoptera (Z).
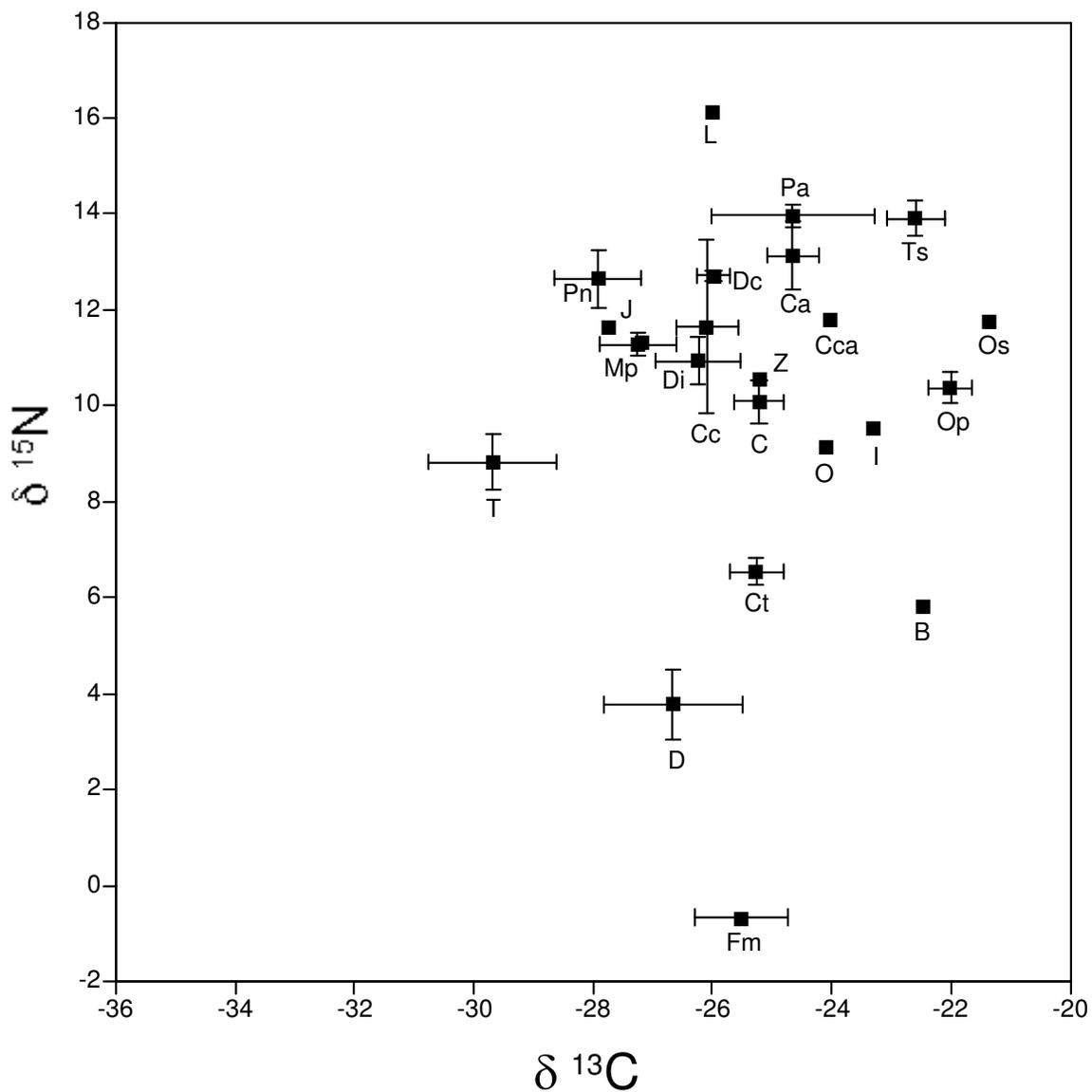
Figure 11. Cave composite graph of temporally and spatially (among site) pooled δ^{13}C and δ^{15}N data, expressed with the following symbology: bacteria (B); *Cambarus tenebrous* (Ct); Chironimidae (C); *Chologaster agassizi* (Ca); *Cottus carolinae* (Cc); *Cyprinus carpio* (Cca); detritus (D); Diptera (Di); *Dorosoma cepedianum* (Dc); fungal mycelia (Fm); Isopoda (I); larval fish (L); *Micropterus punctulatus* (Mp); Oligochaeta (O); *Orconectes pellucidis* (Op); Ostracoda (Os); *Pimephales notatus* (Pn); *Pomoxis annularis* (Pa); tadpole (T); *Typhlichthys subterraneus* (Ts); and Zooplankton (Zo).

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