

Chemolithoautotrophy supports macroinvertebrate food webs and affects diversity and stability in groundwater communities

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Abstract. The prevailing paradigm in subterranean ecology is that below-ground food webs are simple, limited to one or two trophic levels, and composed of generalist species because of spatio-temporally patchy food resources and pervasive energy limitation. This paradigm is based on relatively few studies of easily accessible, air-filled caves. However, in some subterranean ecosystems, chemolithoautotrophy can subsidize or replace surface-based allochthonous inputs of photosynthetically derived organic matter (OM) as a basal food resource and promote niche specialization and evolution of higher trophic levels. Consequently, the current subterranean trophic paradigm fails to account for variation in resources, trophic specialization, and food chain length in some subterranean ecosystems. We reevaluated the subterranean food web paradigm by examining spatial variation in the isotopic composition of basal food resources and consumers, food web structure, stygobiont species diversity, and chromophoric organic matter (CDOM), across a geochemical gradient in a large and complex groundwater system, the Edwards Aquifer in Central Texas (USA). Mean $\delta^{13}\text{C}$ values of stygobiont communities become increasingly more negative along the gradient of photosynthetic OM sources near the aquifer recharge zone to chemolithoautotrophic OM sources closer to the freshwater-saline water interface (FWSWI) between oxygenated freshwater and anoxic, sulfide-rich saline water. Stygobiont community species richness declined with increasing distance from the FWSWI. Bayesian mixing models were used to estimate the relative importance of photosynthetic OM and chemolithoautotrophic OM for stygobiont communities at three biogeochemically distinct sites. The contribution of chemolithoautotrophic OM to consumers at these sites ranged between 25% and 69% of total OM utilized and comprised as much as 88% of the diet for one species. In addition, the food web adjacent to the FWSWI had greater trophic diversity when compared to the other two sites. Our results suggest that diverse OM sources and *in situ*, chemolithoautotrophic OM production can support complex groundwater food webs and increase species richness. Chemolithoautotrophy has been fundamental for the long-term maintenance of species diversity, trophic complexity, and community stability in this subterranean ecosystem, especially during periods of decreased photosynthetic production and groundwater recharge that have occurred over geologic time scales.

Key words: biogeochemical gradient; chemolithoautotrophy; Edwards Aquifer; food web structure; long-term stability; nutrient limitation; phreatic groundwater; resource supply; species richness; stable isotopes; stygobiont; Texas, USA.

INTRODUCTION

Linkages between biodiversity and ecological factors affecting nutrient supply and food web structure have received considerable attention (Levinton 1972, Schoener 1974, Correa and Winemiller 2014). Theoretical and empirical competition studies suggest that species inhabiting low-productivity environments can still exhibit a high degree of specialization and that niche partitioning

reduces competition for limited resources (MacArthur and Pianka 1966, Correa and Winemiller 2014). Even extreme, energy-poor environments have the potential for niche diversification (Fišer et al. 2012). However, specific hypotheses for community-scale trophic structure under various resource supply regimes are difficult to test because most food webs are speciose (Polis and Strong 1996), contain multiple food sources (Phillips and Gregg 2003), and resource utilization can vary with non-trophic, environmental conditions (Fridley et al. 2007).

Subterranean habitats are often touted as ideal natural systems for ecological hypothesis testing because communities comprise a few species that occupy relatively stable habitats with limited potential food resources (Poulson

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and White 1969, Culver et al. 1995, Juan et al. 2010). The prevailing paradigm in speleobiology, largely developed from studies of vadose (unsaturated) zone habitats in accessible caves, suggests that subterranean aquatic food webs are species poor and comprise one or two trophic levels dominated by generalist consumers (Gibert and Deharveng 2002, Hüppop 2012, Poulson 2012, but see Tobler 2008). In these food webs, heterotrophic microorganisms primarily metabolize dissolved organic matter (OM) from the surface (Graening and Brown 2003, Simon et al. 2003). But, allochthonous resource supply in groundwater is spatially and temporally variable, being tightly coupled to surface environmental conditions (i.e.,

photosynthetic productivity and precipitation driven transport) and hydrogeologic structure (i.e., recharge features and groundwater flowpaths).

Over the last 40 years, ecologists have demonstrated that chemolithoautotrophic OM supports or supplements metazoan food webs in diverse aquatic habitats, the most famous being deep-sea, hydrothermal vents (Lonsdale 1977), but also including pelagic marine environments (Swan et al. 2011), deep-sea cold seeps (Paull et al. 1984), lakes (Casamayor et al. 2008), and streams (Kohzu et al. 2004). For groundwater food webs, chemolithoautotrophic OM has largely been considered a minor resource (Gibert and Deharveng 2002, Hüppop 2012, Poulson

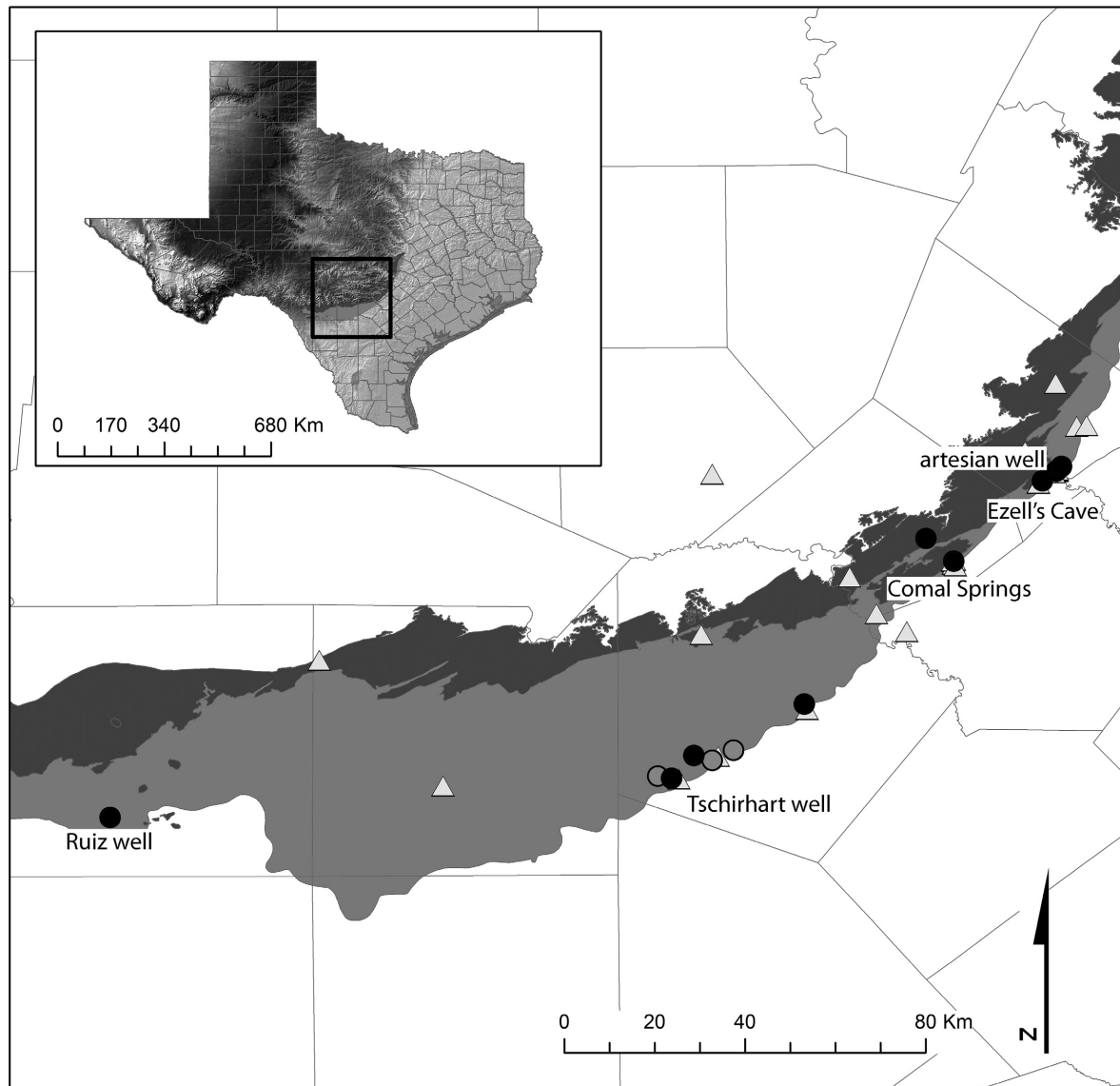


FIG. 1. Study area with major hydrogeologic divisions of the Edwards Aquifer. Dark gray shows the recharge/unconfined zone, and light gray shows the confined zone. Southern and southwestern boundary of confined zone corresponds to the FWSWI. Triangles identify $\delta^{13}\text{C}_{\text{DIC}}$ sampling locations, gray circles indicate $\delta^{13}\text{C}_{\text{DIC}}$ and microbial biomat sampling locations, and black circles indicate $\delta^{13}\text{C}_{\text{DIC}}$ and animal sampling locations. Sampling locations discussed in the text are labeled.

2012), even though some of the most diverse groundwater communities identified (Sarbu et al. 1996, Pohlman et al. 1997, Por 2007) are supported by chemolithoautotrophic production at rates comparable to those reported for epigeal habitats with complex food webs and trophic specialists (Porter et al. 2009), including boreal forests and grasslands (Cramer et al. 2001), lakes (Sabo et al. 2009), and oceanic pelagic zones (Saba et al. 2011). Groundwater food web studies report chemolithoautotrophy in habitats ranging from small cave systems with drainage areas < 10 km² (Sarbu et al. 2000) to the Yucatán Peninsula (Pohlman et al. 1997), which underlies an area > 165 000 km² (Bauer-Gottwein et al. 2011). Chemolithoautotrophy has also been demonstrated for deep aquifers in basalt, granite, and non-carbonate sedimentary rocks (Amend and Teske 2005), although micro- and meiofauna and invertebrate species diversity, food web dynamics, and trophic structures of these systems are practically unknown (Johns et al. 2015). Hypothetically, chemolithoautotrophy in groundwater systems may

control biological and trophic diversity in otherwise species-poor and simple food webs because chemolithoautotrophic OM is a temporally constant resource largely decoupled from surface processes. However, our understanding of how chemolithoautotrophic production affects the evolution and maintenance of complex trophic structure in groundwater ecosystems remains poor.

In this study, we evaluated the relative importance of chemolithoautotrophy among subterranean food webs located in the Edwards Aquifer of central Texas, USA (Fig. 1). The Edwards Aquifer is a large, complex karst aquifer that contains one of the most diverse stygobiont (obligate subterranean aquatic animals) communities (Fig. 2) in the world (Longley 1981). Hutchins and Schwartz (2013) and Hutchins et al. (2014) illustrate that food chain length in the Edwards Aquifer is the longest yet reported from any groundwater system on Earth, containing secondary and potentially higher-level consumers with morphologic adaptations for predation. Moreover, prior research indicates that both

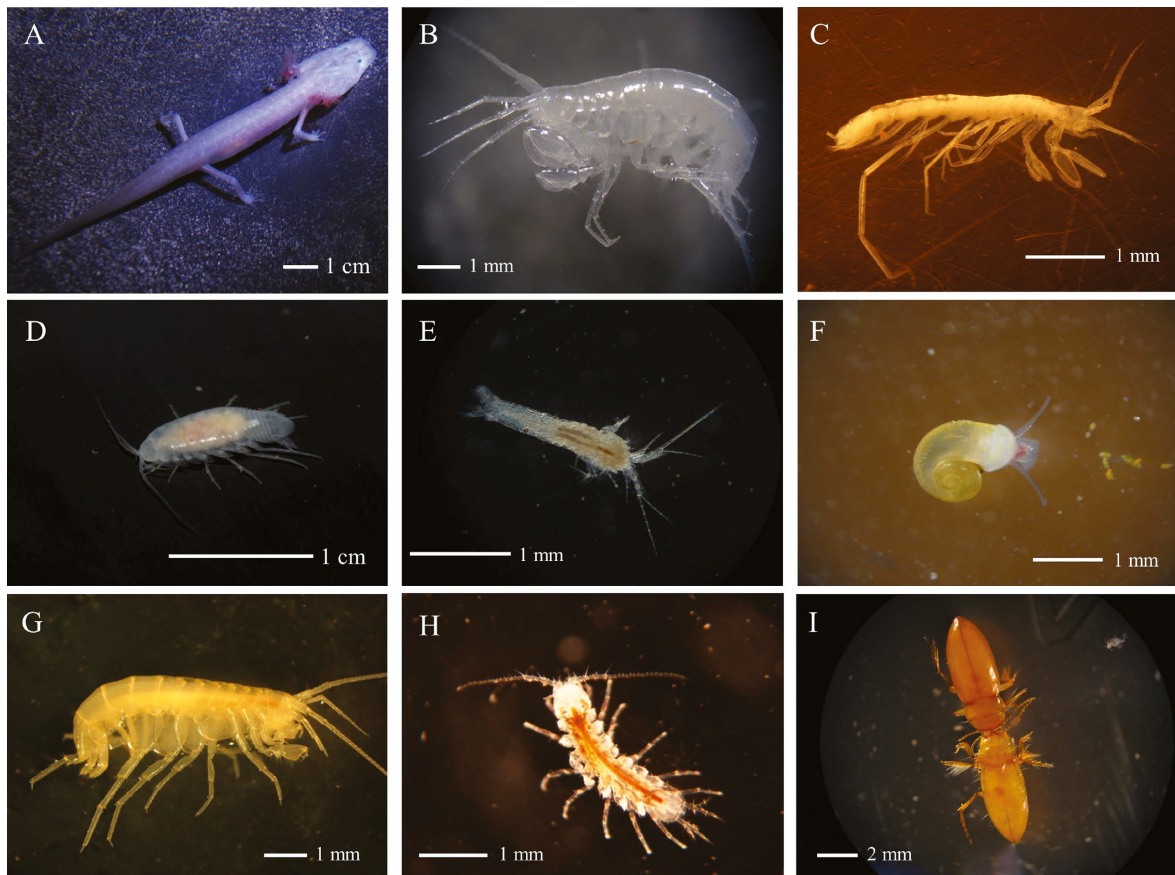


FIG. 2. Images of several Edwards Aquifer stygofauna species found at the artesian well, Comal Springs, and/or Ezell's Cave in Central Texas. (A) Texas blind salamander (*Eurycea rathbuni*; Plethodon), (B) *Artesia subterranea* (Amphipoda), (C) *Parabogidiella americana* (Amphipoda), (D) *Cirolanides texensis* (Isopoda), (E) *Tethysbaena texana* (Thermosbaenacea), (F) domed cavensnail (*Phreatodrobia mugax*; Gastropoda), (G) Peck's cave amphipod (*Stygobromus pecki*; Amphipoda), (H) Texas troglotic water slater (*Lirceolus smithii*; Isopoda), and (I) the Texas cave diving beetle (*Haideoporus texanus*; Coleoptera). Photo credits: panels (A), (B), (E), (F), and (I) by B.F. Schwartz; panels (C) and (G) by B.T. Hutchins; panel (D) by J. Butler; panel (H) by J.R. Gibson (United States Fish and Wildlife Service).

photosynthetic and chemolithoautotrophic OM are locally present but sourced from hydrologically and spatially distinct components of the aquifer (Birdwell and Engel 2009, Gray and Engel 2013, Hutchins et al. 2013). Species richness and food web structure were used to evaluate the existing subterranean food web structure paradigm of pervasive generalist feeding strategies, low trophic complexity, and short food chains. To evaluate potential OM sources supporting stygobiont communities, we examined stable isotope compositions of dissolved inorganic carbon (DIC), fine particulate organic matter (FPOM), dissolved organic matter (DOM), and stygobionts, and we characterized spatial variations in hydrogeochemistry and aquifer chromophoric DOM (CDOM). At three sites, we performed a more detailed analysis of the stable isotope compositions of stygobiont communities to test prevailing hypotheses on subterranean food web structure. Finally, data on stygobiont diversity from across the aquifer were used to assess the potential role of chemolithoautotrophy in maintaining diversity and long-term stability of stygobiont communities. We hypothesized that variable contributions of photosynthetic and chemolithoautotrophic OM to stygobiont food webs are due to spatial variation in hydrogeochemistry. We also hypothesized that species richness positively correlates with proximity to chemolithoautotrophic OM sources because it is produced independently from surface environmental conditions. Finally, we hypothesized that stygobiont food webs utilizing both chemolithoautotrophic and photosynthetic OM (as opposed to only photosynthetic OM) result in food webs that do not adhere to the paradigm of short food chains comprised of generalist consumers.

MATERIALS AND METHODS

Site description

The freshwater portion of the karstic Edwards Aquifer (Fig. 1) spans approximately 360 km along the Balcones Escarpment of Central Texas (Groschen and Buszka 1997) and contains more than 40 stygobiont species. Meteoric water recharges the aquifer and delivers photosynthetically derived OM along the aquifer's north-west margin where surface streams sink in exposed limestone (Maclay and Small 1983, Lindgren et al. 2004). To the south and east, the aquifer is confined up to 1000 m below the surface by clays and marls that prevent recharge (Barker et al. 1994, Lindgren et al. 2004). Along the aquifer's south-east margin, oxygenated, low total dissolved solids (TDS) water from surface recharge is juxtaposed against dysoxic and anoxic, high TDS waters where conductivity increases from 500 $\mu\text{S}/\text{cm}$ to >15 000 $\mu\text{S}/\text{cm}$ and sulfide concentrations can exceed 15 mMol/L (Rye et al. 1981, Groschen and Buszka 1997).

Epilithic microbial mats have been identified in down-bore video logs from saline, sulfidic water wells

(Engel and Randall 2011). Microbial community analyses of mats and water samples indicate that chemolithoautotrophic production occurs in the saline zone and along the FWSWI (Engel and Randall 2011, Gray and Engel 2013), and communities are dominated by sulfur- and methane-oxidizing taxa (Engel and Randall 2011, Gray and Engel 2013) similar to those found in other chemolithoautotrophic karst systems (Porter et al. 2009). Low concentrations of FPOM throughout the aquifer, and especially proximal to the FWSWI, suggest a food source not entrained in the water column (i.e., epilithic microbial mats). CDOM characterization from portions of the aquifer suggests that both photosynthetic and chemolithoautotrophic OM are locally present (Birdwell and Engel 2009).

Spatial variation in geochemistry and potential OM sources across the aquifer

To examine spatial variation in geochemistry and potential chemolithoautotrophic support for stygobiont communities, we collected water, microbial biofilms, and stygobionts from May 2010 to May 2013 at sites distributed across the aquifer. Water samples were collected from 37 sites (Fig. 1) to determine stable carbon isotope ratios of DIC ($\delta^{13}\text{C}_{\text{DIC}}$) and concentrations of dissolved NO_3^- and O_2 (DO). Samples for $\delta^{13}\text{C}_{\text{DIC}}$ were filtered through 0.45 μm Fisherbrand nylon syringe filters, poisoned with sodium azide, and stored in glass vials with butyl rubber septa (Doctor et al. 2008) at 4°C until analysis at the University of California (UC)-Davis Stable Isotope Facility using a GasBench II system interfaced to a Delta V Plus IRMS (Thermo Scientific, Bremen, Germany). For NO_3^- analyses, samples were filtered through 0.45 μm Fisherbrand nylon syringe filters and stored in the dark at 4°C until measurement with a Dionex ICS-1600 ion chromatograph (Bannockburn, Illinois, USA). DO was measured in the field with an optical DO sensor on a Troll® 9500 sonde (In-Situ, Inc., Fort Collins, Colorado, USA).

A subset of samples ($n = 18$), including from locations not previously analyzed by Birdwell and Engel (2009), were taken for CDOM analysis by filtering water through 0.2 μm Whatman PVDF filters and storing in the dark at 4°C until analysis at the University of Tennessee-Knoxville, Department of Earth and Planetary Sciences, following methods of Birdwell and Engel (2009). Detailed procedures are described in Appendix S1. The biological or autochthonous index (BIX) was determined from the ratio of fluorescence intensity at 380 nm to that at 430 nm at the excitation wavelength of 310 nm from corrected EEM spectra (Birdwell and Engel 2009, 2010, Hugué et al. 2009). This index was used as a measure of the contribution of microbially-based OM, with values between 0.8 and 1 indicative of fresh DOM of biological or microbial origins and values <0.6 indicative of OM sourced from terrestrial humics (Birdwell and Engel 2010).

Microbial biofilm samples ($n = 7$) were collected from three sites. Microbial biofilm samples were not easily obtained because of difficulty accessing and sampling deep artesian wells that intersect the FWSWI and because epilithic biofilms are infrequently entrained in the water column. Thus, the number of samples we could collect was limited. Microbial mats were divided into two subsamples; one placed in a fuming HCl chamber for 24 h to remove inorganic carbon prior to $\delta^{13}\text{C}$ analysis, and one left untreated for nitrogen isotope ($\delta^{15}\text{N}$) analysis. FPOM was collected from six surface streams that recharge the aquifer, with collection, processing, and analyses following Hutchins et al. (2013). Although bacterivorous protozoans have the potential to link microbial production to higher trophic levels via the “microbial loop” (Azam et al. 1983), macroinvertebrates in a variety of aquatic systems have repeatedly been shown to graze directly on epilithic, microbial biofilms (Lear et al. 2012). Furthermore, the role of protozoans in groundwater is more poorly known (Novarino et al. 1997) and protozoan fauna in the Edwards Aquifer are completely unstudied. For these reasons, as well as practical limitations associated with collecting sufficient material for isotopic analysis, an assessment of protozoans in the Edwards Aquifer food web was beyond the scope of this study.

Two hundred and ninety-two individual stygobionts of 23 species were collected using baited traps (animals were prevented from feeding on the bait), drift nets, and/or hand collection from nine sites across the aquifer: two springs, two caves, and five wells. The two springs (San Marcos and Comal Springs) are major discharge features with discrete openings fed by deeply-circulating groundwater. Sampling was accomplished by placing drift nets in or over spring openings so that animals collected were discharged directly from the aquifer and not residing in the benthos or hyporheos of the spring run. Once collected, organisms were kept alive in filtered spring water for ~ 3 h. For smaller sized species, 2–42 individuals were combined prior to isotopic analysis to assure adequate sample mass. Snails were removed from shells prior to analysis. Samples from the Texas blind salamander (*Eurycea rathbuni*) were taken from the base of the tail. All biological samples were dried at 50°C for 48 h, homogenized, and sent to the UC-Davis Stable Isotope Facility for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Comparison of food webs that differ in chemolithoautotrophic production

To examine variability in the contribution of photosynthetic and chemolithoautotrophic OM to stygobiont communities at different locations across the aquifer, and potential OM source effects on food web structure, three sites were chosen for more detailed analysis. Sample

dates, preparation, and isotope analytical methods were as described earlier. Comal Springs, Ezell’s Cave, and an artesian well on the campus of Texas State University, San Marcos, Texas, were chosen to span a gradient between photosynthetic OM and chemolithoautotrophic OM dominated food webs, for ease of access for sampling, and because high stygobiont numbers allowed more thorough sampling of the invertebrate community. At all three sites, suspended fine particulate organic matter (FPOM) samples were collected for $\delta^{13}\text{C}$ analysis (Comal Springs $n = 13$, Ezell’s Cave $n = 2$, artesian well $n = 3$). Low FPOM concentrations (< 1 mg/L) precluded $\delta^{15}\text{N}$ analysis.

Data analysis

To examine OM resource spatial variability in groundwater food webs in the Edwards Aquifer, we assessed relationships between stygobiont community isotopic compositions ($\delta^{13}\text{C}_{\text{spp}}$) and variables related to large-scale hydrological and biogeochemical gradients indicative of spatial proximity to potential chemolithoautotrophic and photosynthetic OM inputs. In addition to geographic proximity to the FWSWI and recharge zone, we used concentrations of electron acceptors (O_2 and NO_3^-) to provide an additional measure of hydrologic proximity to the recharge zone and the FWSWI. Dominant electron acceptors (O_2 and NO_3^-) originate in the recharge zone and concentrations decrease towards the FWSWI due to combined effects of microbial decomposition of photosynthetic OM (Katz et al. 2004), denitrification (Arango et al. 2007, Burgin and Hamilton 2007), and enhanced NO_3^- reduction coupled with sulfide oxidation during chemolithoautotrophic carbon fixation along the FWSWI (Burgin and Hamilton 2008, Gray and Engel 2013).

We used ordinary least-squares, multiple linear regressions to evaluate relationships between C isotopic composition of animal tissues and microbial mats ($\delta^{13}\text{C}_{\text{spp}}$) and the aforementioned geochemistry across 13 sites (Appendix S1, Fig. 1). Akaike Information Criterion (AIC) was used to select from competing models. Spatial and geochemical variables from the best model predicting $\delta^{13}\text{C}_{\text{spp}}$ values were used as predictors of CDOM BIX values and $\delta^{13}\text{C}_{\text{DIC}}$ values in additional ordinary least-squares, multiple linear regressions. We predicted that $\delta^{13}\text{C}_{\text{spp}}$ compositions would become more negative with decreasing availability of dominant electron acceptors and increasing distance from the recharge zone (i.e., increasing proximity to the FWSWI). If changes in stygobiont C isotope signatures were the result of changes in the relative importance of chemolithoautotrophic vs. photosynthetic OM production, then BIX values would also positively correlate with proximity to the FWSWI and negatively correlate to the summative concentration of the major free electron acceptors. Furthermore, we predicted that $\delta^{13}\text{C}_{\text{DIC}}$ values should become less negative with increasing proximity to the FWSWI as a result of

TABLE 1. Multiple regression results from analysis of $\delta^{13}\text{C}$ values of animals and dissolved inorganic carbon and the biological/ autochthonous index (BIX) of chromophoric dissolved organic matter versus proximity to the freshwater-saline water interface (FWSWI) and the availability of dominant electron acceptors (summation of molar concentration).

Response variable	Whole model				log distance FWSWI		Concentration of O_2 and NO_3^-	
	r^2	P	F	df	Coefficient†	P	Coefficient†	P
$\delta^{13}\text{C}_{\text{spp}}$ (‰)	0.68	0.003	12.49	2,9	3.979 ± 1.265	0.012	33.612 ± 8.900	0.004
$\delta^{13}\text{C}_{\text{DIC}}$ (‰)	0.62	<0.001	30.53	2,34	0.010 ± 0.098	0.917	-16.461 ± 2.484	<0.001
BIX	0.71	<0.001	19.44	2,13	-0.041 ± 0.008	<0.001	-0.208 ± 0.147	0.180

Note: Bold P values indicate significance at $\alpha = 0.05$.

†Coefficients \pm standard error.

(1) degassing of isotopically light CO_2 (Deuser and Degens 1967) in the unconfined portion of the aquifer in the recharge zone, (2) atomic exchange with bedrock (Gonfiantini and Zuppi 2003) that increases with residence time in the confined and saline portions of the aquifer (Lindgren et al. 2004), and (3) a transition from carbonic acid dissolution near the recharge zone to sulfuric acid dissolution near the FWSWI (Engel and Randall 2011, Gray and Engel 2013). Carbonic acid is generated from elevated CO_2 levels due to the decomposition of soil OM near the recharge zone, and sulfuric acid results from chemolithoautotrophic microbes that oxidize reduced sulfur compounds near the FWSWI. Finally, we predicted that $\delta^{13}\text{C}_{\text{spp}}$ values would be inversely correlated with $\delta^{13}\text{C}_{\text{DIC}}$ values. Increasingly negative $\delta^{13}\text{C}_{\text{spp}}$ values, indicative of a chemolithoautotrophic food source, were expected to correspond with positive $\delta^{13}\text{C}_{\text{DIC}}$ values resulting from bedrock dissolution driven by sulfuric acid produced near the site of chemolithoautotrophic production.

We used quantile regression to test whether species richness was a function of proximity to the FWSWI. Quantile regression was appropriate for this analysis because we expected uneven variance in species richness at different distances from the FWSWI because of variation in aquifer connectivity, local geology and geochemistry, and because of differences in sampling effort among sites that could influence species richness regardless of proximity to the FWSWI. Significance was assessed at the 50th, 75th, and 95th percentiles (Cade and Noon 2003). Statistical analyses were performed in R v3.0 (additional statistical methods in Appendix S1).

For the more detailed food web analysis for Comal Springs, Ezell's Cave, and the artesian well, we first evaluated whether animals were feeding as generalists by testing whether species occupy the same position in trophic space (as defined by $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values) using multivariate analysis of variance (MANOVA) and Fisher's LSD post-hoc tests of separate ANOVAs of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were used to define putative trophic groups used in Bayesian mixing models. Because we selected these sites to span a gradient between photosynthetic and chemolithoautotrophic OM input to the food webs, we quantified relative food source

contributions for stygobionts and to bulk, suspended FPOM from each site using Bayesian mixing models in the R package, SIAR v4.2 (Parnell and Jackson 2013). The $\delta^{13}\text{C}$ values of top consumers (identified by $\delta^{15}\text{N}$ values) at sites were used to quantify the relative importance of OM source to the entire food web because consumers integrate carbon from disparate OM sources utilized by lower trophic level consumers (Jepsen and Winemiller 2002). Photosynthetic, surface stream FPOM, chemolithoautotrophic FPOM, and stygobiont trophic groups were used as potential food sources for consumers in mixing models. The chemolithoautotrophic end-member was estimated using three separate methods (model design detailed in Appendix S1).

RESULTS

Of 11 evaluated models, AIC suggested that mean $\delta^{13}\text{C}_{\text{spp}}$ values for stygobionts and microbial mats across the aquifer were best predicted by a combination of geographic proximity to the FWSWI and O_2 and NO_3^- concentrations (Table 1; Appendix S1: Table S1), with $\delta^{13}\text{C}_{\text{spp}}$ values becoming more negative with increasing proximity to the FWSWI and with decreasing availability of O_2 and NO_3^- . The combination of these predictor variables also defined a gradient between terrigenous, humified and humic-like CDOM indicative of photosynthetic OM at sites proximal to recharge features to unhumified proteinaceous CDOM indicative of chemolithoautotrophic OM near the FWSWI (Table 1). Mean $\delta^{13}\text{C}_{\text{DIC}}$ values were a significant negative function of the summative concentration of the major free electron acceptors in the water (Table 1). Lastly, quantile regression revealed that stygobiont biodiversity decreased with increasing distance from the FWSWI and source of chemolithoautotrophic OM at the 95th and 75th percentiles (Fig. 3).

At the three biogeochemically distinct sites examined for food web structure, 75 individuals from 10 species were collected at Comal Springs, 35 individuals from six species were collected from Ezell's Cave, and 141 individuals from 16 species were collected from the artesian well (Appendix S1: Table S2). Passive sampling, low detectability of organisms, and lack of direct access to

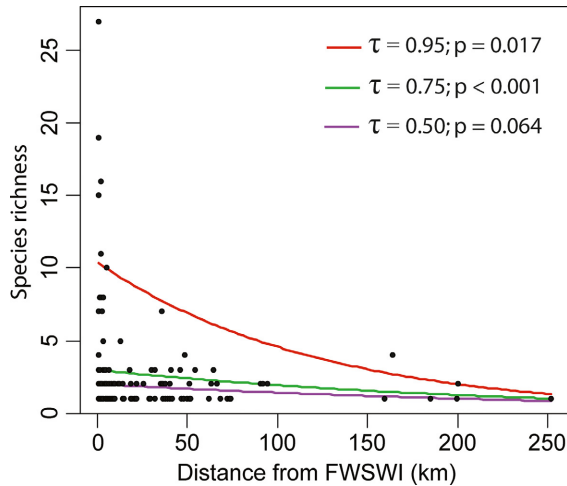


FIG. 3. Quantile regression of species richness as a function of distance from the freshwater saline water interface (FWSWI). Trendlines are shown for significant quantiles (τ).

the deep aquifer limited the sample size for some of the groundwater invertebrates. Nevertheless, to our knowledge, our data represent the largest stable isotope analysis of any stygobiont assemblage in terms of sample size, number of species, and geographic extent surveyed (reviewed in Hutchins and Schwartz 2013).

Across the three sites, changes in the relative contributions of photosynthetic OM and chemolithoautotrophic OM were apparent, as indicated by $\delta^{13}\text{C}_{\text{spp}}$ compositions (Fig. 4). MANOVA indicated significant differences among $\delta^{13}\text{C}_{\text{spp}}$ and $\delta^{15}\text{N}$ values for the different species, which were confirmed by isotope-specific ANOVAs (Appendix S1: Table S2). Based on significantly different $\delta^{13}\text{C}_{\text{spp}}$ values, two distinct food chains at the artesian well were apparent (Fig. 4, Appendix S1: Table S3). Results from Bayesian mixing models incorporating all measured $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic data suggested that the posterior probability estimate (ppe) for the contribution of chemolithoautotrophic OM to animal diets (ppe-COM) at the artesian well site was as great as 88% (95% equal-tailed credible intervals = 82–94%) for some species, and as little as 35% (4–58%) for others

(Fig. 4A, Table 2). For the top consumer, *Artesia subterranea*, ppe-COM is 69% (56–78%) and carbon was assimilated entirely through predation on intermediate consumers (Appendix S1). $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{13}\text{C}$ values from microbial mats suggested that chemolithoautotrophic

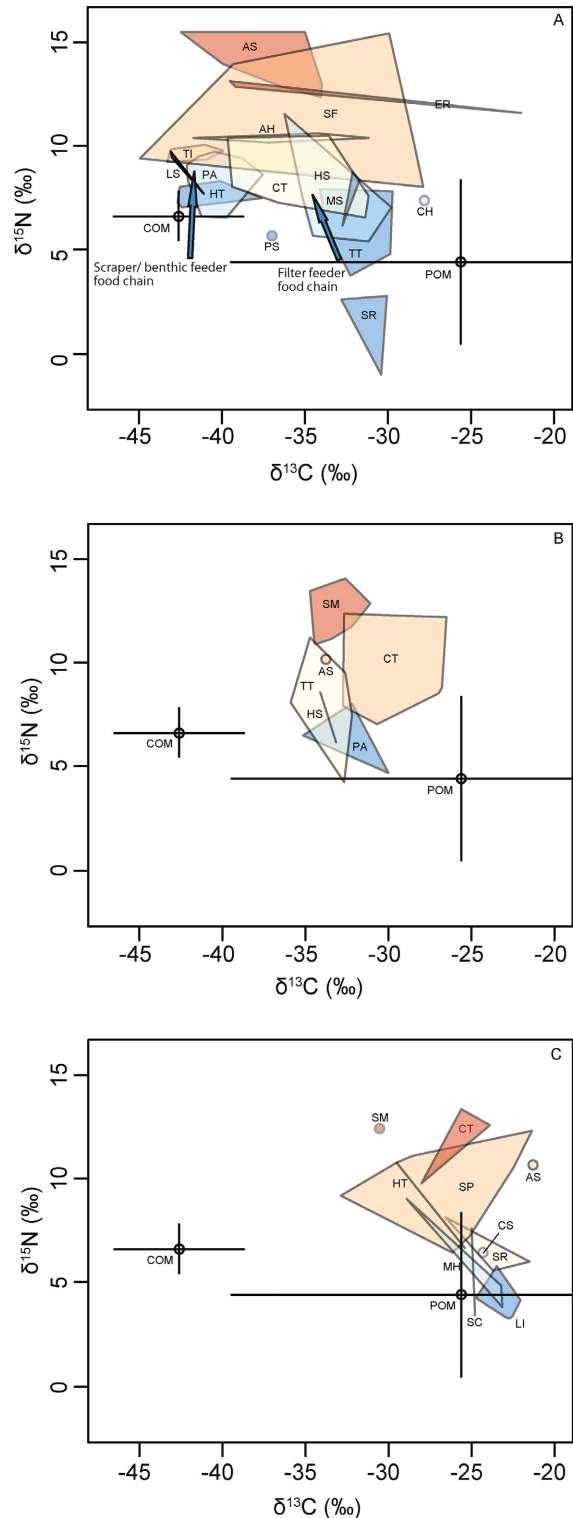


FIG. 4. Isotope biplots for stygobionts from (A) artesian well, (B) Ezell's Cave, and (C) Comal Springs. Polygons are convex hulls encompassing all data points for species. N is 1 for species shown by small circles. AH is *Allotexiweckelia hirsuta*; AS is *Artesia subterranea*; CH is *Calathaemon holthuisi*; CS is *Comaldessus stygius*; CT is *Cirolanides texensis*; ER is *Eurycea rathbuni*; HS is *Holsingerius samacos*; HT is *Haedioporus texanus*; LS is *Lirceolus smithii*; LI is *Lirceolus* spp.; MH is *Mexiweckelia hardeni*; MS is *Moorbdella* sp.; PA is *Palaemonetes antrorum*; PS is *Phreatodrobia* spp.; SC is *Stygoparnus comalensis*; SF is *Stygobromus flagellatus*; SM is *Sphalloplana mohri*; SP is *Stygobromus pecki*; SR is *Stygobromus russelli*; TI is *Texiweckeliopsis insolita*; TT is *Texiweckelia texensis*; COM is chemolithoautotrophic organic matter; POM is photosynthetic organic matter.

TABLE 2. Posterior probability estimates (ppe) of the contribution of food sources to consumers (95% equal tail confidence intervals) via direct consumption.

Consumer	Sources	Posterior estimate
Artesian well <i>Artesia subterranea</i>	<i>S. flagellatus</i> group	0.44 (0.13–0.71)
	<i>C. texensis</i>	0.16 (0.01–0.44)
	Scraper/benthic forager group	0.27 (0.03–0.49)
	Filter feeder group	0.07 (0.00–0.29)
	<i>S. russelli</i>	0.03 (0.00–0.14)
<i>Eurycea rathbuni</i>	<i>S. flagellatus</i> group	0.29 (0.02–0.57)
	<i>C. texensis</i>	0.18 (0.01–0.48)
	Scraper/benthic forager group	0.34 (0.05–0.65)
	Filter feeder group	0.09 (0.00–0.33)
	<i>S. russelli</i>	0.05 (0.00–0.21)
<i>Stygobromus flagellatus</i>	<i>C. texensis</i>	0.45 (0.13–0.77)
	Scraper/benthic forager group	0.25 (0.03–0.49)
	Filter feeder group	0.12 (0.01–0.37)
	<i>S. russelli</i>	0.02 (0.00–0.09)
	COM	0.06 (0.00–0.24)
<i>Allotexiweckelia hirsuta</i>	POM	0.04 (0.00–0.15)
	<i>C. texensis</i>	0.22 (0.03–0.41)
	Scraper/benthic forager group	0.23 (0.04–0.42)
	Filter feeder group	0.16 (0.01–0.35)
	<i>S. russelli</i>	0.07 (0.00–0.22)
<i>Texiweckeliopsis insolita</i>	COM	0.20 (0.02–0.38)
	POM	0.10 (0.01–0.27)
	Scraper/benthic forager group	0.35 (0.08–0.59)
	Filter feeder group	0.06 (0.00–0.28)
	<i>S. russelli</i>	0.05 (0.00–0.18)
<i>Lirceolus smithii</i>	COM	0.47 (0.19–0.77)
	POM	0.03 (0.00–0.18)
	Scraper/benthic forager group	0.31 (0.03–0.64)
	COM	0.66 (0.34–0.92)
	POM	0.03 (0.00–0.12)
<i>Cirolanides texensis</i>	Scraper/benthic forager group	0.25 (0.06–0.42)
	Filter feeder group	0.17 (0.01–0.35)
	<i>S. russelli</i>	0.17 (0.04–0.30)
	COM	0.27 (0.09–0.45)
	POM	0.15 (0.01–0.29)
<i>Palaemonetes antrorum</i>	COM	0.88 (0.82–0.94)
	POM	0.12 (0.05–0.18)
	<i>Holsingerius samacos</i>	<i>T. texensis</i>
<i>Moorbdella sp.</i>	<i>S. russelli</i>	0.21 (0.02–0.43)
	COM	0.32 (0.17–0.46)
	POM	0.21 (0.02–0.40)
	<i>T. texensis</i>	0.26 (0.03–0.51)
	COM	0.28 (0.03–0.57)
<i>Haedioporus texanus</i>	COM	0.25 (0.05–0.43)
	POM	0.21 (0.02–0.44)
	COM	0.92 (0.82–0.99)
<i>Texiweckelia texensis</i>	POM	0.08 (0.01–0.18)
	COM	0.43 (0.19–0.57)
	POM	0.57 (0.43–0.81)
<i>Phreatodrobia spp.</i>	COM	0.54 (0.10–0.89)
	POM	0.46 (0.11–0.90)
	COM	0.35 (0.04–0.58)
<i>Calathaemon holthuisi</i>	POM	0.65 (0.42–0.96)
	COM	0.30 (0.04–0.49)
	POM	0.70 (0.51–0.96)
FPOM	COM	0.30 (0.04–0.49)
	POM	0.70 (0.51–0.96)

Table 2 (Continued)

Consumer	Sources	Posterior estimate
Ezell's Cave		
<i>Sphalloplana mohri</i>	<i>T. texensis</i> group	0.50 (0.21–0.77)
	<i>P. antrorum</i>	0.21 (0.02–0.48)
	<i>C. texensis</i>	0.27 (0.03–0.55)
<i>Cirolanides texensis</i>	<i>T. texensis</i> group	0.29 (0.05–0.49)
	<i>P. antrorum</i>	0.31 (0.05–0.57)
	COM	0.08 (0.00–0.24)
	POM	0.32 (0.18–0.46)
	COM	0.53 (0.31–0.69)
<i>Texiweckelia texensis</i>	POM	0.47 (0.31–0.69)
	COM	0.46 (0.11–0.72)
<i>Palaemonetes antrorum</i>	POM	0.54 (0.28–0.89)
	COM	0.31 (0.04–0.52)
FPOM	POM	0.69 (0.48–0.96)
Comal Springs		
<i>Sphalloplana mohri</i>	<i>S. pecki</i> & <i>H. texanus</i>	0.77 (0.26–0.99)
	<i>M. hardeni</i> group	0.23 (0.01–0.74)
<i>Cirolanides texensis</i>	<i>S. pecki</i> & <i>H. texanus</i>	0.34 (0.05–0.68)
	<i>M. hardeni</i> group	0.30 (0.03–0.60)
	COM	0.11 (0.01–0.37)
	POM	0.22 (0.01–0.50)
<i>Artesia subterranea</i>	<i>S. pecki</i> & <i>H. texanus</i>	0.42 (0.03–0.91)
	<i>M. hardeni</i> group	0.58 (0.09–0.97)
<i>Stygobromus pecki</i>	<i>H. texanus</i>	0.32 (0.22–0.42)
	<i>M. hardeni</i> group	0.35 (0.22–0.51)
	COM	0.14 (0.10–0.18)
	POM	0.19 (0.05–0.30)
<i>Haedioporus texanus</i>	<i>M. hardeni</i> group	0.38 (0.04–0.75)
	COM	0.27 (0.03–0.60)
	POM	0.35 (0.03–0.70)
<i>Stygobromus russelli</i>	<i>Lirceolus</i> sp. & <i>M. hardeni</i>	0.45 (0.08–0.86)
	COM	0.11 (0.01–0.45)
	POM	0.40 (0.05–0.75)
<i>Comaldessus stygius</i>	<i>M. hardeni</i> & <i>Lirceolus</i> spp.	0.43 (0.05–0.84)
	COM	0.13 (0.01–0.37)
	POM	0.42 (0.06–0.77)
<i>Mexiweckelia hardeni</i>	<i>Lirceolus</i> spp.	0.51 (0.13–0.87)
	COM	0.10 (0.01–0.34)
	POM	0.37 (0.03–0.73)
<i>Stygoparnus comalensis</i>	<i>Lirceolus</i> spp.	0.43 (0.05–0.84)
	COM	0.18 (0.01–0.56)
	POM	0.36 (0.03–0.74)
	COM	0.07 (0.00–0.22)
<i>Lirceolus</i> sp.	COM	0.93 (0.78–1.00)
	POM	0.34 (0.05–0.67)
FPOM	POM	0.66 (0.33–0.95)
Ruiz well		
<i>Cirolanides texensis</i>	COM	0.08 (0.00–0.25)
	POM	0.92 (0.75–1.00)

Notes: COM, chemolithoautotrophic organic matter; POM, photosynthetic organic matter; artesian well *S. flagellatus* group: *Stygobromus flagellatus*, *Eurycea rathbuni*, and *Artesia subterranea*; artesian well scraper/benthic forager group: *Texiweckeliopsis insolita*, *Haedioporus texanus*, *Palaemonetes antrorum*, and *Lirceolus smithii*, artesian well filter feeder group: *Holsingerius samacos*, *Moorbdella* sp. and *T. texensis*; Ezell's Cave *T. texensis* group: *Texiweckelia texensis*, *Holsingerius samacos*, and *Artesia subterranea*, Comal *M. hardeni* group: *Mexiweckelia hardeni*, *Lirceolus* sp., *Stygobromus russelli*, *Comaldessus stygius*, and *Stygoparnus comalensis*.

OM constitutes a higher proportion of total OM at some other sites (e.g., Tschirhart well, Fig. 1), but we were unable to collect animals at these sites. Therefore, the artesian well represents the most chemolithoautotrophic OM-dependent site for which we presently have food web data. Ezell's Cave, an intermediate site along the photosynthesis-chemolithoautotrophy OM gradient, receives some terrestrial input through the entrance and as bat guano, but the cave also intersects phreatic groundwater near the FWSWI. Species-specific ppe-COM ranged between 37% (23–50%) and 53% (31–69%) (Fig. 4B, Table 2). For the top consumer, *Sphalloplana mohri*, ppe-COM was 47% (32–60%) and assimilation of chemolithoautotrophic OM was entirely through predation. Comal Springs is a near-surface site overlain by highly fractured limestone that facilitates photosynthetic OM input as detritus and living roots. The ppe-COM was between 7% (0–22%) and 31% (22–50%) for individual species at Comal Springs, and the ppe-COM for the top consumer, *Cirolanides texensis*, was 25% (11–46%) and derived from a combination of predation and direct consumption of chemolithoautotrophic OM (Fig. 4C, Table 2). *Cirolanides texensis* at Ruiz well (Fig. 1) appeared to assimilate less chemolithoautotrophic OM, with a ppe-COM of 10% (1–27%), although sample size was small and no additional species could be collected. Raw data for all analyses are included in Data S1.

DISCUSSION

The prevailing paradigm of subterranean food web structure describes short food chains based on spatially and temporally heterogeneous food resources that result in species-poor communities comprised of generalist consumers. The Edwards Aquifer clearly does not adhere to this paradigm because of the presence of diverse stygobiont communities that include predators and trophically-specialized consumers. Cumulatively, dissolved inorganic constituent patterns (Groschen and Buszka 1997), $\delta^{13}\text{C}_{\text{DIC}}$ values (Hutchins et al. 2013), $\delta^{13}\text{C}_{\text{spp}}$ values, species richness patterns, and microbial community structure in the FWSWI (Engel and Randall 2011, Gray and Engel 2013) suggest that food web structure in the Edwards Aquifer is controlled by a gradient from photosynthetic-dominated food webs near recharge features to chemolithoautotrophic-dominated food webs near the FWSWI. Although sample size was limited, spatial variation in CDOM properties across the aquifer also support this conclusion. Fixation of inorganic carbon was not directly measured, for example, by using radiotracers, but our interpretations are consistent with conclusions of observed changes in microbial community structure near the FWSWI (Engel and Randall 2011, Gray and Engel 2013) and conclusions of Birdwell and Engel (2009) that assessed microbial community composition and CDOM to argue that OM

near the FWSWI was likely produced *in situ* from chemolithoautotrophy.

Chemolithoautotrophic primary production near the FWSWI and multiple food resources overall sets the stage for increased horizontal and vertical trophic complexity in the Edwards Aquifer. Horizontal trophic complexity is apparent as distinct aquatic food chains based on phototrophy and chemolithoautotrophy. We argue that these food chains are dominated by, respectively, filter feeding species and grazer/benthic foraging species that utilize epilithic microbial mats. Hutchins et al. (2014) demonstrate that amphipod species from the artesian well that have $\delta^{13}\text{C}_{\text{spp}}$ values suggestive of a chemolithoautotrophic food source also exhibited mouthpart morphology consistent with scraping, but species with $\delta^{13}\text{C}_{\text{spp}}$ values suggestive of a photosynthetic food source exhibited mouthpart morphology consistent with filter feeding. Similarly, the decapods *P. antrorum* and *C. holthuisi* have $\delta^{13}\text{C}_{\text{spp}}$ values consistent with chemolithoautotrophic and photosynthetic food sources, respectively, and their mouthpart morphologies are also consistent with benthic foraging and filter feeding, respectively (Bruce and Short 1993, Cooper and Cooper 2011). Horizontal trophic diversity increases the amount of biomass available to support higher trophic levels (Duffy et al. 2007, Cardinale et al. 2009), which could lengthen the food chain and have the expected result of secondary and potentially higher-level consumers with morphologic adaptations for predation, reported by Hutchins and Schwartz (2013) and Hutchins et al. (2014).

Environmental stability also plays a role in food chain length (Sabo et al. 2010). Subterranean ecosystems are often cited as being well-buffered against environmental variability, but subterranean communities may be sensitive to long-term changes in surface environmental conditions if dependent upon inputs of surface resources such as photosynthetic OM. Geologic, paleontological, and archaeological data reveal repeated episodes of pronounced aridity in Texas (Holliday 1989, Otvos 2004, Ellwood and Gose 2006) since the end of the Late Miocene. During these episodes, decreased recharge and resource input could have reduced groundwater population sizes and potentially caused local extirpations of some species dependent solely on photosynthetic OM. However, we hypothesize that chemolithoautotrophic primary productivity in the aquifer was decoupled from climate change effects that would otherwise affect some subterranean systems (Dobrowski 2011), and a persistent chemolithoautotrophic OM resource may have led to decreased extinction rates by increasing population sizes and ranges relative to “islands” of favorable habitat centered around discrete photosynthetic OM inputs (Mittelbach et al. 2007). Indeed, the presence of marine-derived fauna (i.e., Hadziid and Sebid amphipods, Cirolanid isopods, and Thermosbaenacea) that likely colonized the Edwards

Aquifer during the Paleocene-Eocene thermal maximum (Fisher and McGowen 1967, Yancey et al. 2012) or earlier (Holsinger and Longley 1980) is evidence for long-term persistence of species in the system, despite variability in photosynthetic OM availability over time.

That chemolithoautotrophy enables portions of the Edwards Aquifer to serve as an evolutionary refugium is also evidenced by the modern-day decrease in Edwards Aquifer stygobiont biodiversity with increasing distance from the FWSWI (Fig. 3), which could be due to either lower extinction rates (see above) or higher diversification rates near the FWSWI relative to the recharge zone. Diversification through allopatric (Mittelbach et al. 2007) and sympatric speciation (Bolnick 2004) can be promoted through increased resource constancy and supply rate, both of which are consequences of chemolithoautotrophy. Although neither extinction rates nor diversification rates were explicitly assessed in this study, both could result in food chains with increased trophic complexity and higher diversity of local competitors (Cardinale et al. 2009), as observed in the Edwards Aquifer. More generally, the presence of chemolithoautotrophy should be an important driver of metazoan diversity in any system characterized by heterogeneous and limiting nutrient supply, such as deep-sea, cold seep communities (Sibuet and Olu 1998).

Given the diversity of groundwater ecosystems in which chemolithoautotrophy has been identified, its role in groundwater habitats is likely more widespread than currently acknowledged. Indeed, Por (2007) hypothesized the presence of a globally distributed and contiguous, chemolithoautotrophy-based biome in the subsurface. But, there is currently a substantial knowledge gap about the extent and importance of chemolithoautotrophy in subterranean, groundwater food webs.

We conclude that chemolithoautotrophic OM production in the Edwards Aquifer has increased available food resources for stygobionts and has provided a constant, long-term nutrient supply that has promoted evolution of horizontal trophic diversity and increased food chain length. Chemolithoautotrophic primary productivity is fundamental to the evolution and maintenance of biodiversity and trophic complexity in groundwater habitats, which may be important as many groundwater systems are being threatened by drought (Dobrowski 2011) and groundwater pumping that exceeds recharge rates (Gleeson et al. 2012). Stygobionts are characterized by a suite of life history traits and ecological characteristics that result in increased sensitivity to habitat degradation (Culver et al. 2000, Culver and Pipan 2009), but preservation efforts targeting groundwater habitats are still in their infancy (Michel et al. 2009). Consequently, an understanding of food web structure and mechanisms that promote and maintain biodiversity in the subsurface is increasingly important.

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