Methane- and dissolved organic carbon-fueled microbial loop supports a tropical subterranean estuary ecosystem

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Subterranean estuaries extend inland into density-stratified coastal carbonate aquifers containing a surprising diversity of endemic animals (mostly crustaceans) within a highly oligotrophic habitat. How complex ecosystems (termed anchialine) thrive in this globally distributed, cryptic environment is poorly understood. Here, we demonstrate that a microbial loop shuttles methane and dissolved organic carbon (DOC) to higher trophic levels of the anchialine food web in the Yucatan Peninsula (Mexico). Methane and DOC production and consumption within the coastal groundwater correspond with a microbial community capable of methanotrophy, heterotrophy, and chemosynthesis, based on characterization by 16S rRNA gene amplicon sequencing and respiratory quinone composition. Fatty acid and bulk stable carbon isotope values of cave-adapted shrimp suggest that carbon from methanotrophic bacteria comprises 21% of their diet, on average. These findings reveal a heretofore unrecognized subterranean methane sink and contribute to our understanding of the carbon cycle and ecosystem function of karst subterranean estuaries.
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cognition that chemosynthetic biological communities capture energy and synthesize organic matter (OM) from chemicals emitted from the seafloor1–3 and the oceanic carbon cycle4. OM produced by chemosynthetic pathways and expelled from seafloor hydrothermal vents and cold seeps may exceed 10% of surface ocean productivity4, 5. Submarine groundwater discharge is another important source of nutrients and carbon to the ocean6, in some instances exceeding that of rivers7, and therefore represents a critical exchange vector between continental land-masses and the ocean8. However, much more is known about the magnitude of submarine groundwater discharge to the coastal ocean than the genesis of the material expelled9, 10. Evidence that a coastal aquifer food web in the Yucatan peninsula (YP) (Mexico) is partially dependent on a chemosynthetic food source10 suggests mutual biogeochemical reactions govern the ecology of these Earth–Ocean transition zones, yet the basic carbon cycle in this widely distributed coastal aquifer ecosystem remains largely unexplored. In the present study, we adapt methods previously used to investigate continental-margin cold seeps11–13 to delineate the biogeochemistry and functional ecology of this coastal aquifer.

Mixing of terrestrial meteoric water with saline groundwater (SGW) in coastal aquifers resembles the two-layered circulation of surface estuaries such that they have been termed subterranean estuaries14. Subterranean estuaries are found globally along siliciclastic, basaltic, and karstic (carbonate) coastlines15, 16, 17. The most prevalent and human-accessible estuary type is found within porous limestone of karst coastlines, where marine-derived groundwater extends inland beneath the meteoric lens flooding extensive cave passages16, 17. Karst coastlines account for ~25% of coastlines globally15 and ~12% of all submarine groundwater discharge9. Research conducted by scientific divers within cave conduits of coastal aquifers has led to a basic understanding of stygobitic (cave-limited) macrofaunal biodiversity18, 19, within this globally distributed ecosystem (termed anchialine, meaning near the sea)10, the food web structure10, how sea level change during the Holocene affected the habitat development20, and hydrologic controls that govern the mixing of fresh and marine waters within the subterranean estuary17.

The seminal investigation of anchialine ecosystems suggested OM supporting consumers of the food web in a tropical subterranean estuary was partially derived from a chemosautrophic source10. This conclusion was based on the bulk stable carbon isotopic composition of several crustacean species that were distinct (13C-depleted) from available photosynthetic sources and similar to invertebrates from deep sea vent communities that rely on a chemosautrophic food base. Comparable isotopic values were reported for invertebrates from a thermoninal cave in Romania with clear evidence that mantle-derived hydrogen sulfide (H2S) was the primary energetic source22. The anchialine ecosystem investigated by Pohlman et al.10 contained no H2S or other evidences of mantle derived material, suggesting that non-sulfurous reduced compounds (e.g., ammonium or methane) liberated during OM decomposition support microbial communities. However, they were unable to definitively constrain the nutritive OM source. Subsequent studies from a freshwater karst aquifer23, a sunlit anchialine sinkhole24, an alluvial aquifer25, and freshwater lakes26 also suggest higher trophic level invertebrates utilize chemosautrophic products generated from OM degrada-

dating, suggesting the possibility that ecosystems deep within a coastal aquifer are sustained by similar processes.

In this study, we investigated the carbon cycle and food web dynamics of a pristine anchialine ecosystem within a tropical karst subterranean estuary in Mexico’s YP. The YP is a limestone platform that contains more than 1000 km of mapped cave conduits within the coastal region of the Holbox fracture zone27 (Fig. 1). These cave passages prevail within the inland portion of the subterranean estuary over an area (~1100 km2) comparable to surface estuaries like Galveston Bay (Texas) (~1500 km2), the 7th largest estuary in the U.S. Natural sinkholes, locally known as cenotes, provide scientists direct access to the flooded caves. The site we investigated (Cenote Bang) is located ~8 km inland within a mature dry tropical forest, and is one of the entrances to the Ox Bel Ha cave network (Fig. 1c; Supplementary Fig. 1).

Based on the observation that a complex food web exists in coastal karst aquifers with limited particulate OM, we tested the hypothesis that dissolved organic carbon (DOC)—including methane—formed from decomposition of terrestrial vegetation within water saturated limestone beneath the tropical forest provides carbon and energy for a microbial loop that, in turn, supports the subterranean food web28. We identified carbon sources and inferred biogeochemical cycles based on the distribution, concentration, and isotopic composition of organic and inorganic carbon compounds, and electron acceptor availability. We characterized the microbial community by sequencing of 16S ribosomal RNA (rRNA) genes and identifying quinone lipid biomarkers from environmental water samples. To link the microbes to the food web, we performed compound-specific isotopic analysis of membrane-derived fatty acids (FAs) extracted from filter-feeding cave-adapted shrimp. This multifaceted study provides a broad perspective for carbon transformations and exchange between the terrestrial and marine realms of a tropical karst subterranean estuary.

Results

Water column properties. To characterize the physical and chemical environment of caves accessed from Cenote Bang (Fig. 1), we collected sonde profiles during four sampling campaigns (Fig. 2) between 2013 and 2016 in the Ox Bel Ha Cave System. For all events, we observed three distinct water masses separated by thin (20–60 cm) haloclines (H1 and H2) that were relatively constant in depth (Fig. 2). Salinity in the layer nearest the cave ceiling of the shallowest passages (~3 m water depth) ranged from 0.3 to 0.7 psu, which was slightly less than the cenote pool (0.9–1.8 psu). Salinity ranged from 2.0 to 2.5 psu in the middle layer, and from 34.8 to 37.6 psu in the deepest layer. Sampling of the deep SGW was restricted to 22 m depth below groundwater table due to the geometry of the cave passages. To differentiate the subterranean water masses, we hereafter refer to the low salinity water mass as meteoric freshwater (MFW), the intermediate salinity water mass as meteoric brackish water (MBW), and the deep water layer underlying the meteoric lens as SGW. Moreover, we refer to the coastal sea water as SEA and the open-to-air sinkhole/cenote as POOL, recognizing that the POOL is part of the meteoric lens (Fig. 1e).

Dissolved oxygen in the MFW was at or near anoxia (0–15 µM) and constant in the vertical extent for each campaign. The SGW displayed the highest dissolved oxygen (DO) content (45–55 µM) (Fig. 2), but was still always hypoxic (<60 µM). The MBW showed two distinct profile types. During August 2014 and January 2015, DO was mostly invariant with depth in the MBW (22–29 µM). By contrast, during December 2013 and January 2016, MBW was anoxic near the shallow halocline (H1) and increased gradually with increasing depth toward the deeper halocline (H2). During the days preceding the sonde profilings, there was substantially more rainfall in December 2013 (457 mm) and January 2016 (253 mm) than during August 2014 (52 mm) and January 2015 (39 mm) (Supplementary Table 1). DO in the POOL was consistently low (10–37 µM), but always elevated relative to the MFW and MBW during each event.
Aqueous biogeochemistry. To investigate the distribution, sources, and turnover of dissolved and particulate carbon in the water masses, we collected 63 samples from the three water layers and analyzed them for concentration and δ13C values of dissolved inorganic carbon (DIC), particulate organic carbon (POC), DOC, and methane (CH4), as well as sulfate and chloride concentrations (Supplementary Data 1). Table 1 provides average values for each parameter measured during the course of the study as a baseline for characteristic concentrations and carbon isotopic contents for each water mass, including the open-to-air cenote pool and the coastal ocean (additional details in Supplementary Tables 2, 3).

The anoxic-MFW had the highest methane concentrations (3550–9522 nM) with δ13C-CH4 values (~66.3 ± 0.7‰; Fig. 3a, b; Supplementary Fig. 2) that are characteristic of microbial methane29. Methane concentrations in the hypoxic-MBW (43–275 nM) were lower than those from the POOL (100–890 nM), and about an order of magnitude less than the MFW. MBW (~52.7 ± 1.9‰) and pool (~50.6 ± 4.9‰) δ13C-CH4 values were similar to each other, but substantially more 13C-enriched than observed for the MFW. The δ13C-CH4 values from December 2013, following a period of exceptional precipitation (Supplementary Table 1), were the most 13C-enriched. The hypoxic-SGW had the lowest methane concentrations in the aquifer (37–208 nM) and were similar to the coastal sea values (43–235 nM). The δ13C-CH4 values in the SGW (~56.3 ± 1.5‰) were comparable to those in nearby coastal ocean waters (~59.0 ± 2.1‰) and were 13C-enriched relative to the MFW. Compared to the concentration and carbon isotopic ranges predicted from conservative (non-reactive) mixing models that use MFW and deep SGW methane end member values (Fig. 3a, b), the intermediate depth MBW CH4 concentrations were lower and δ13C-CH4 values were higher, indicating methane removal by oxidation29.

Like methane, DOC concentrations were highest in the anoxic-MFW (402–834 μM), an order of magnitude lower in the MBW (37–203 μM), and lowest in the deep SGW (15–80 μM; Fig. 3c, d;
Table 1 Aqueous biogeochemistry

|                              | Meteoric freshwater | Meteoric brackish water | Saline groundwater SGW | Sinkhole (cenote) POOL | Coastal water SEA POOL |
|------------------------------|---------------------|-------------------------|-------------------------|------------------------|------------------------|
| Salinity (psu)               | 0.26 ± 0.03 (8)     | 1.81 ± 0.04 (29)       | 32.87 ± 0.94 (13)      | 0.94 ± 0.09 (6)        | 35.45 ± 0.39 (6)       |
| [SO₄²⁻] mM                   | 0.3 ± 0.17          | 1.6 ± 0.1 (27)         | 26.4 ± 1.0 (11)        | 0.8 ± 0.1 (6)          | 28.4 ± 0.6 (4)         |
| [CH₄] nM                     | 6466 ± 659 (8)      | 157 ± 16 (28)          | 110 ± 17 (12)          | 495 ± 148 (6)          | 121 ± 28 (6)           |
| δ¹³C-CH₄ %e                  | −66.3 ± 0.7 (7)     | −52.7 ± 1.9 (25)       | −56.3 ± 1.5 (11)       | −50.6 ± 4.9 (6)        | −59.0 ± 2.1 (5)        |
| [DOC] μM                     | 661 ± 132 (3)       | 131 ± 16 (16)          | 41 ± 20 (3)            | −              | −              |
| δ¹³C-DOC %e                  | −28.0 ± 0.1 (3)     | −28.3 ± 0.2 (16)       | −26.6 ± 0.4 (3)        | −              | −              |
| [DIC] mM                     | 4.4 ± 0.6 (6)       | 7.1 ± 0.2 (24)         | 2.4 ± 0.2 (10)         | 5.3 ± 0.2 (3)         | 2.0 ± 0.1 (3)          |
| δ¹³C-DIC %e                  | −16.4 ± 1.0 (7)     | −11.3 ± 0.7 (25)       | −6.3 ± 1.0 (11)        | −9.4 ± 2.1 (3)        | −4.3 (2)               |
| POC μM                       | 10.9 ± 3.8 (3)      | 5.0 ± 2.3 (3)          | 3.0 ± 0.9 (3)          | 32.3 ± 14.9 (3)       | 5.8 (1)               |
| δ¹³C-POC %e                  | −28.5 ± 0.5 (3)     | −27.6 ± 0.7 (3)        | −27.1 ± 1.0 (3)        | −28.0 ± 0.3 (3)       | −20.1 (1)              |

Values of water column constituents, presented as average ± std. error (n), from the different regimes of the groundwater system and the adjacent coastal sea. Values were calculated from all measurements within a water mass across all sampling events. For further information regarding data obtained during the separate sampling events, see the supplement (Supplementary Tables 2, 3).

Fig. 3 Plots of salinity vs. chemical properties from the subterranean estuary. a Methane concentrations. b Dissolved organic carbon (DOC) concentrations. c Methane stable carbon isotopic (δ¹³C) values. d Methane stable carbon isotopic (δ¹³C) values. f DIC stable carbon isotopic (δ¹³C) values. The average and total area of conservative mixing lines (CMLs; see Methods for calculations) represent the trend predicted by the mixing model if there was only physical mixing between the meteoric freshwater and saline groundwater end members. Production yields an excess of the constituent relative to the CML average and area, while consumption results in depletion. Symbol of individual data points contain the uncertainty (1 std. dev.) of the measured values.

Supplementary Fig. 2). The MFW δ¹³C-DOC values (−28.0 ± 0.1‰) were consistent with a terrestrial C3 plant origin,³⁰ the dominant vegetation in the overlying tropical forest. The SGW δ¹³C-DOC values (−26.6 ± 0.4‰) were slightly higher due to contributions from the coastal ocean.³¹ Similar to methane, DOC concentrations in the MBW were much lower when compared to predictions from the conservative mixing model, indicating removal of DOC (Fig. 3c, d). However, for August 2014, the majority of the DOC samples displayed low δ¹³C values (Fig. 3d), opposite of the effect expected for oxidation. Consistent with the distribution of DOC, the highest POC concentrations occurred in the anoxic-MFW (3.3–14.6 μM). However, POC does not contribute significantly to the total organic carbon pool, with average concentrations only 1.6% of the DOC. The δ¹³C-POC values in the MFW (−28.5 ± 0.5‰) are consistent with a forest vegetation origin.³⁰

DIC was the largest pool of carbon in the cave waters (Fig. 3c, f; Supplementary Fig. 2). Biological respiration, carbonate
dissolution, and chemolithotrophic CO₂ assimilation have the potential to alter DIC concentration and carbon isotopic ratios. High concentrations of DIC in the MBW require the addition of DIC from a 13C-enriched source. The most likely source for such a large input of DIC is dissolution of carbonate, which occurs within this groundwater mixing zone and has δ13C values that are ~0%[2]. Sulfate, a potential electron acceptor for OM respiration and/or the anaerobic oxidation of methane, did not vary in concentration relative to the conservative mixing diagram (Supplementary Fig. 3).

**Microbial community**. To characterize the microbial community structure in the density-stratified aquifer, the open-water cenotes, and the coastal ocean, we sequenced 16S rRNA genes and analyzed respiratory quinone biomarkers from January 2016 water samples (Fig. 4). Phylogenetic affiliations were assigned based on 16S rRNA gene sequences and were clustered into metabolic groups by inferred phenotypes of representative sequences within each operational taxonomical unit. These functional groups were determined to identify microbes capable of mediating biogeochemical pathways inferred from geochemical analyses (Fig. 4a). Because the water samples were collected within the water masses and not at the interfaces between water masses (where we hypothesize carbon consumption to be most active), the sequence data are a qualitative indicator of the microbial community composition. Nevertheless, the cenote pool, MFW, and MBW showed relatively high abundances of sequences from type I (1.2–2.3%) and type II (<0.3%) methanotrophic bacteria, sulfur-oxidizing bacteria, and other archaeal and bacterial functional groups involved in methylotrophy, as well as chemoautotrophic nitrogen and sulfur cycling processes. The relative abundance of methanogenic archaea was below 1%, with highest abundance in the SGW, where ammonia-oxidizing microbes were also present (Fig. 4). Numerous other microbes capable of utilizing a wide range of organic compounds were identified (“Others” in Fig. 4; Supplementary Fig. 4; Supplementary Data 2).

Quinone biomarkers offer DNA-independent detection and quantification of microbial biomass in samples from the natural environment. In our samples, the occurrence and relative distribution of quinones were distinct for the meteoric and saline water regimes (Fig. 4b). The major quinone types in all samples were ubiquinones (UQs) containing 7–10 isoprenoid units and 1–2 double bonds per isoprenoid unit (see quinone nomenclature in Methods). Additionally, in the samples from the POOL, the MFW and MBW methylene-ubiquinone (MQ8:7) was detected, which structurally differs from regular UQs by the presence of a methylene group in the isoprenoid side chain. In the samples from the POOL and MFW, UQ8:8 was the dominant quinone (72% relative abundance), while in the samples from the MBW, UQ8:8 and UQ9:9 contribute 40% and 41% to total quinones, respectively. UQ8:8, UQ9:9, and UQ10:10 were equally distributed in the deep SGW, while UQ10:10 was the dominant quinone with number of double bonds. MBW, meteoric brackish water; MFW, meteoric freshwater; POOL, cenote pool; SEA, coastal sea; SGW, saline groundwater.
62% in the coastal sea water. Highest quinone concentrations occurred in the POOL with 31 ng l⁻¹. In the cave, the concentrations decreased with increasing salinity across the different water regimes (MFW, MBW, SGW) (Fig. 4b). The quinone MQ₈:₇ is diagnostic for type II methanotrophs and UQ₉:₉, but the latter is also widespread among other aerobic bacteria36, but the latter is also widespread among other aerobic bacteria36, but the latter is also widespread among other aerobic bacteria36. The SGW and coastal ocean (SEA) samples, in contrast, mainly contained UQ₉:₉ and UQ₁₀:₁₀, which occur in diverse aerobic bacteria.

**Bulk stable isotopes.** To determine the trophic relationships between potential food sources and consumers, specimens (n = 29) of stygobitic *Typhlatya* spp., a free-swimming atyid shrimp with feeding appendages capable of capturing bacteria-sized particles10, 37 and insects (n = 4) from the surface jungle, were measured for stable carbon and deuterium isotopic content (Fig. 5a). Twelve shrimp were obtained from the MBW of Cenote Bang cave. The remaining specimens (n = 17) were collected from the MBW and SGW of three locations connected (via cave conduits) with the main research site and two caves at greater distance that are not likely linked to Cenote Bang (Supplementary Fig. 5). Shrimp stable carbon isotope values ranged from −22.5 to −49.1‰, and the stable hydrogen isotope values ranged from −95.7 to −223.6‰ (Fig. 5a). These isotope values were between the terrestrial soil/insect values and those expected for microbial methane. The measured δ¹³C-CH₄ values from the cave were typical for microbial methane, and the δ¹³C-DOC values were similar to those of the soil OM.

**Fatty acid biomarkers.** We report fractional abundances and stable carbon isotope values of FAs extracted from two shrimp specimens (collected from Cenote Bang) with relatively small (3%) and large (55%) contributions of methane carbon to the specimen’s biomass, as calculated from a two-source mixing model25, 38. We observed a range of C₁₄–C₁₈ FAs, all of which displayed δ¹³C values similar to the specimen’s bulk δ¹³C (Fig. 5b; Supplementary Table 4). FA compounds extracted from the shrimp with relatively high methane contribution to its biomass (Shrimp 1; Fig. 5b) displayed more negative δ¹³C values than FAs from the tissue of Shrimp 2 (Fig. 5b). Both shrimp contained generic, saturated FAs with an even number of carbon atoms (C₁₄₀, C₁₆₂, and C₁₈₂), as well as odd number unsaturated and methylated lipid compounds.

**Discussion**

The results presented above demonstrate that methane and DOC derived from degraded terrestrial OM are the primary carbon and energy sources for a karst submarine estuarine ecosystem beneath an undisturbed tropical forest (Fig. 6). Variability in the DO profiles (Fig. 2b) and carbon chemistry (Fig. 3) of the water column suggests external factors influence the spatial and temporal dynamics of the aquifer biogeochemistry. However, the emphasis of this study and the following discussion is to identify unifying characteristics for developing a generic model of ecosystem function for this terrestrially influenced submarine estuary to be applied to other anchialine ecosystems.

The most basic physical characteristic for this coastal aquifer and others24 is the uniform and extreme density stratification of the 25 m water column. The three distinct water masses separated by two sharp haloclines were present in the cave conduits during all sampling campaigns (Fig. 2a). The physicochemical characteristics of the MFW in the cave were distinct from the POOL, which had slightly higher salinity (~ 1.0 psu) and...
oxygen (10–37 μM) contents. MFW occurs throughout the permeable karst aquifer (Fig. 1e); however, access to that portion of the aquifer was restricted to shallow, domed cave passages that extend vertically upward to water depths of 5 m or less. Herein, we argue the MFW is of critical importance to the carbon cycle of terrestrially influenced habitats in the anchialine ecosystem.

The shallowest portion of the aquifer is in contact with soil OM within saturated fissures and pores of the carbonate rock. Anaerobic decomposition of this soil OM from the overlying tropical forest vegetation, as indicated by its stable carbon isotopic composition (δ13C = −28.5‰), is consistent with a microbial methanogenesis (CH4) value (−66.3‰) is consistent with a microbial methanogenesis (CH4) value (−66.3‰) is comparable to the MFW, DOC is the primary source of carbon sediments, and the water column of lakes and oceans are driven by pulses of sinking particulate detritus produced in surface waters or within the watershed basin. The accumulation and consumption of organic detritus depletes oxygen below the chemocline. For OM oxidation to continue, oxygen or alternate electron acceptors (e.g., sulfate, nitrate, etc.) must be replenished by mixing. By contrast, in the tropical karst aquifer we investigated, depleted oxygen (Fig. 2b) co-occurred with concentrated methane (Fig. 3a) and DOC (Fig. 3c) above the shallow chemocline (H1). The relatively high concentrations of oxygen in the deepest sampled portion of the aquifer (SGW) is consistent with the transport of DO with sea water moving inland from the coast below the deeper halocline (H2 in Fig. 2). Distinct DO profiles preceded by periods of high and low rainfall (Fig. 2b) suggest precipitation is the key external factor regulating electron acceptor availability in the meteoric portion of the aquifer. We hypothesize that rainfall injects oxygenated water into the MBW at discrete entrances by point recharge, and drives DOC-enriched water from the anoxic saturated portion of the aquifer (the MFW) into the caves by diffuse recharge.

Previous studies in caves suggest POC concentrations are limited in karst groundwater. To evaluate POC bioavailability and origin in this coastal karst aquifer, we measured concentrations and δ13C values of POC for June 2015 and January 2016 (Supplementary Table 3) and compared them to DOC concentrations and δ13C values in the cave environment. Like DOC, POC is most abundant in the MFW (10.9 μM) and derived from the tropical forest vegetation, as indicated by its stable carbon isotopic composition (δ13C = −28.5‰). However, on average, DOC in the MFW is 60 times more abundant than POC. By comparison, DOC:POC ratios range between 6 and 10 in the surface ocean, rivers, and streams. In the oligotrophic Atlantic Ocean, where DOC:POC ratios from 300 m water depth are comparable to the MFW, DOC is the primary source of carbon.
available to a microbial loop that supports the pelagic ecosystem. We conclude, as others have for cave streams, some riverine systems and oligotrophic oceans, that DOC is a more important source of carbon and energy than POC for this coastal aquifer ecosystem.

Carbon-based concentration and isotopic mixing models (Fig. 3) provide insight into evaluating if and where different carbon stocks are created or consumed within a mixing system. This approach has been used to investigate carbon dynamics in estuaries and high-salinity marine pore waters. We applied this concept to this subterranean estuary, and calculate using equation (4) that, on average, 93% of the methane (Fig. 3a) and 76% of the DOC (Fig. 3c) were removed within the MBW. The methane concentration reduction was 6300 nM and the DOC reduction was 530 µM, on average, suggesting there is an active sink for methane and bulk DOC. During oxidation of methane and DOC, CO₂ is certainly produced, however, production of 13C-enriched DIC in the MBW resulting from carbonate dissolution (Fig. 3e, f) overwhelms the isotopic effect from the respired CO₂.

Enrichment of 13C in the methane within the MBW is consistent with microbial oxidation being the removal mechanism. Due to the enzyme oxidation of methane, there is a bias toward utilization of the 12C-isotope, leaving the residual methane 13C-enriched, as observed here. By contrast, although the concentration-based mixing model for DOC indicates removal (Fig. 3c), a large positive carbon isotopic shift was not observed for the December 2014 data (Fig. 3d). This observation does not, however, conflict with the model evidence because isotopic fractionation of DOC during aerobic oxidation is less than what occurs during methane oxidation. The negative shift in the δ13C of DOC for the August 2014 sampling event suggests production of DOC from methane carbon. Conservative mixing of sulfate during all sampling events (Supplementary Fig. 3) indicates that sulfate reduction did not considerably contribute to the dissolved OM oxidation, but this analysis may not be sufficiently sensitive to detect changes of sulfate relative to carbon pools with orders of magnitude lower concentrations. The presence of DO in the MBW is additional evidence that methane and DOC oxidation were aerobic.

Analyses of microbial community structure (16S rRNA) and respiratory quinones reveal a diverse microbial community with distinct structuring within the karst subterranean estuary, the open-water cenote and the coastal ocean (Fig. 4; Supplementary Fig. 4; Supplementary Data 2). Sequences representing microbes that consume methane, utilize sulfur-and nitrogen-based electron acceptors to oxidize OM, as well as sulfur-oxidizers were relatively abundant in the freshwater portion of the aquifer (MFW) and sinkhole (POOL). In particular, type I methanotroph sequences from the genus Methylococcaceae were present in the POOL, as well as the MFW and MBW water masses, where geochemical data clearly indicate methane oxidation (Fig. 3).

The presence of respiratory quinones, which are lipid-soluble components of the electron transport chain, provide evidence for metabolically active microbial functional groups in the subterranean estuary. The most prevalent quinones are affiliated with aerobic heterotrophic bacteria (Fig. 4b), which is consistent with the metabolic capacity of most microbes observed ("Others" in Fig. 4a) and with DOC being the most abundant form of OM consumed. In the meteoric water masses, the predominant quinone was UQ₈, which only occurs in strictly aerobic and facultatively anaerobic (grown under aerobic conditions) organisms and is the dominant quinone in type I methanotroph cultures. This compound peaked in abundance in the MFW and POOL locations, where we also found highest 16S rRNA gene copy numbers of type I methanotrophs (Fig. 4). Detectable concentration of the quinone biomarker MQ₈, which has only been found in type II methanotrophs, was also present in the portion of the groundwater where methane was oxidized. The dominance of type I over type II methanotrophs is not surprising, because they are generally more prevalent in environments with low oxygen, like those observed in this subterranean estuary, and are more efficient at converting methane carbon to biomass than are type II methanotrophs.

Sequences from numerous genera that mediate chemotrophic carbon fixation and utilization through oxidation and reduction of sulfur- and nitrogen-based compounds were also present in the open-air cenote and cave (Fig. 4). However, we presently have no evidence that these microbes contribute to the carbon cycle or food web of the cave. A sulfate mixing model similar to the carbon mixing models (Fig. 3) did not indicate removal of sulfate in the cave (Supplementary Fig. 3). Furthermore, none of the passages we investigated contained detectable H₂S. By contrast, deep open-water cenotes found in the YP, where organic debris accumulates near the deeper halocline (H2 in Fig. 2a) are most certainly settings where the carbon and sulfur cycles are intertwined. Microbes from those areas may have been transported into the interior of the YP limestone platform. Alternatively, a cryptic sulfur cycle is active or the mixing model lacks the sensitivity required to detect changes in sulfate concentration. Nitrification within the mixing zone of the MBW and SGW has been suggested as another potential chemotrophic source of OM in a YP anchialine ecosystem. Near this interface, we found the coexistence of ammonia oxidizers typically found in either marine (Nitrosopumilus) or terrestrial environments (Nitrososphaera) (Fig. 4). However, given the relatively low concentrations of nitrate (18.6 µM) accumulated near the MBW–SGW interface relative to the amount of DOC consumed (530 µM), and the low carbon assimilation efficiency of nitrifying bacteria, the likelihood that nitrification contributes meaningful nutritive carbon to the food web remains speculative. Nevertheless, the sequence data are consistent with the hypothesis that multiple biogeochemical cycles utilizing all available electron donors and acceptors are active in these oligotrophic and anoxic/hypoxic habitats. Additional studies are required to evaluate their importance for the food web. Our data support that DOC (including methane) derived from decomposition of terrestrial OM is the prevalent source of nutritive carbon that sustains the ecosystem.

Bulk stable carbon and hydrogen isotopic data from Typhlatya spp. shrimp adapted to feed on bacteria-sized, suspended matter in the water column are consistent with a mixed dietary dependence on methane- and DOC-derived carbon (Fig. 5a) via the consumption of microbial biomass. Considering the range of shrimp tissue δ13C values (−23 to −49‰), and the average MFW δ13C values of methane (−65.3‰) and DOC (−28.0‰) (Table 1) as potential end members of the shrimp’s dietary carbon source, the contribution of methane carbon for the shrimp ranges from 0 to 55%, with an average contribution of 21% (Supplementary Table 5). Studies from a humic lake and an alluvial aquifer report methane carbon contributions to zooplankton and insects ranging from 5 to 67%. Because the low δD values in the shrimp are distinctive for methane carbon incorporation, we used the shrimp bulk isotope values to estimate the δD signature of the methanogenesis. By extrapolating the carbon and deuterium stable isotope values from the cave shrimp to the average MFW δ13C-C₄H₄ source value (−66.3‰), we estimated the δD-CH₄ signature was about −390‰, which allowed us to constrain that the microbial methane was produced by acetoclastic methanogenesis. Methane and DOC-derived carbon flow into the anchialine food web is facilitated by trophic interactions between the shrimp.
and its microbial food source. To examine the biochemical origins of the δ 13C-depleted values of the bulk shrimp tissue, we analyzed the composition and carbon isotopic content of FA biomarkers extracted from shrimp with the largest (55%) and smallest (3%) calculated methane contributions (Fig. 5b; Supplementary Table 4). The composition and δ 13C values of FAs differed substantially between the two shrimp, which is consistent with assimilation of different food and carbon sources. In addition, in both specimens, individual FAs differed in their stable carbon isotopic composition, which is indicative of different biochemical pathways for FA synthesis. Even-chain saturated FAs (C14:0, C16:0, and C18:0) had compound-specific δ 13C values closely matching the bulk tissue (Fig. 5b), suggesting the shrimp synthesised these compounds de novo from their dietary carbon sources. In contrast, several monounsaturated FAs in both shrimp and methylated FAs in shrimp 1, showed substantially more negative δ 13C values than the bulk tissue (Fig. 5b), which provides evidence for a dietary source of these compounds through the direct transfer of FAs from ingested bacteria. Given the strong geochemical evidence for aerobic methane oxidation in the hypoxic environment, we attribute the source of stable carbon isotope signatures observed here. A symbiotic source of the incorporated bacterial biomass might also explain the observed FA profiles. However, Typhlatya shrimp appendages capable of direct filter-feeding of bacteria-sized particles suggest the methanotrophic biomass is incorporated from the environment rather than from symbiotic sources. These observations have significant implications for understanding energy transfer within the anchialine food web. Considering that Typhlatya spp. are one of the most abundant macrofaunal populations in this habitat, and they are the primary prey for predators in the subterranean food web, it is reasonable to assume they have a significant role in transferring methane- and DOC-derived carbon to higher levels of the food web.

The geochemical, genomic, and biomarker evidence from this study supports the hypothesis that a microbial loop is active in a karst subterranean estuary ecosystem (Fig. 6). We demonstrated that DOC and methane produced from soil OM degradation within the shallow, anoxic saturated zone of the karst are transported downwards into hypoxic cave conduits, where methanotrophs and heterotrophs consume these reduced OM forms and co-exist with a host of chemoautotrophs. The presence of FAs in somatic tissues of filter-feeding shrimp that could only originate from microbes is strong evidence that microbial biomass is directly transferred to higher-order metazoans. This microbial loop is unique from that of the oligotrophic oceans in that it contains a methane sink, but is likely to be similar to other groundwater systems, where evidence for a similar biogeochemistry has been reported. The generic model of ecosystem function presented here provides baseline information for future studies aiming to quantify the magnitude of this unaccounted for “upside-down” methane sink and to describe the external factors that alter the internal biogeochemical cycling of subterranean estuaries within karst coastal aquifers.

**Methods**

**Study sites and seasons.** Between 2013 and 2016, five field campaigns were conducted to investigate flooded cave networks accessible through Cenote Bang (the primary study site; Fig. 1; Supplementary Fig. 1) within the Ox Bel Ha Cave System and secondary locations (Supplementary Fig. 5). A comprehensive listing of <2-km maps1) and advancing with the probes projecting forward to ensure an undisturbed profile of the water column.

**Sample collection and processing.** Water samples for geochemical analysis of dissolved materials were collected near the sonde profile locations in plastic 60 ml syringes fitted with 3-way stopcocks. The syringes were rinsed with distilled water and dried prior to the dive, and flushed with sample water prior to closing the stopcock. Samples for POC and lipid analyses were collected in 10 liters collapsible nylon bags. Each water sample was kept cool during transport to the field lab and processed within 8 h of collection. Samples for aqueous geochemistry were handled and stored as indicated in Supplementary Table 6. Among those, the serum vials for methane measurement were prepared prior to sample collection by heating water at a concentration of 0.2 ml 1 M NaOH into the empty vial, sealing the container with a 1 cm thick butyl septa, and vacuuming the vial of air with a pump. The water sample was then transferred through the septum with a 20-gauge syringe needle. Water samples for POC, lipid, and rRNA analyses were vacuum filtered through 47 mm diameter polycarbonate filters (Gelman GF/PM 70 lpm mesh or GF/PM 130 lpm mesh for 13C-depleted marine bottom waters) and frozen on dry ice, and then stored in the laboratory at −20 °C.

**Geochemical analysis.** Geochemical analyses were performed at the Woods Hole Oceanographic Institution (WHOI) and U.S. Geological Survey (USGS) in Woods Hole, MA, USA. Headspace methane concentrations were determined using a Shimadzu 14-A gas chromatograph (GC) equipped with a flame ionization detector. Methane was isothermally (50 °C) separated from other headspace gases with a Poraplot-Q stainless steel column (8 ft x 1/8” OD) packed with 60/80 mesh and quantified against certified gas standards with a relative standard deviation (RSD) of 2.8% or less. Headspace concentrations were converted to dissolved concentrations using the method of Magen et al. The stable carbon isotope composition of methane from the headspace of the sample vials was measured using a Thermo-Finnigan DELTAPlus XL isotope ratio mass spectrometer (IRMS) coupled to an Agilent 6890 Gas Chromatograph (GC) via a Finnigan GC/C/combustion interface. Variable volume (1–15 ml) gas samples, depending on concentrations, were introduced through a gas sampling valve into a 1 ml min−1 He stream. methane and other gases were trapped on fused silica capillary packed with 80/100 mesh Poraplot-Q immersed in liquid nitrogen. The gases were thermally desorbed from the column at 150 °C and separated on a 30 m, 0.32 mm ID Poraplot-Q column at −40 °C prior to being oxidized to CO2 and analyzed by IRMS. The δ 13C/δ 18O ratios of methane are expressed in the standard δ notation using the standard δ notation using the standard (VPDB) standard. The carbon and oxygen isotope standard (1) of a 1% CH4 standard analyzed at least eight samples was 0.3%.

For the DOC subsamples, 1:1000 tracer metal grade 12 N HCl/H2O volume ratio was added prior to analysis to achieve pH < 2. DOC concentration and δ 13C were analyzed by high-temperature combustion-isotope ratio mass spectrometer (HTC-IRMS) at the USGS-WHOI dissolved carbon isotope lab (DCIL). The DCIL HTC-IRMS system consists of an OI 1030C total carbon analyzer and a Graden HTC-IRMS system.
molecular sieve trap interfaced to a Thermo-Finnigan DELTAplus XL IRMS. Stable carbon isotope ratios are reported in the standard δ notation relative to VPDB and were corrected by mass balance to account for the analytical blank, which was less than the equivalent of 15 μM DOC in the sample. By comparison, the blank-corrected sample DOC concentrations ranged from 15.3 to 851 μM. Thus, the blank correction ranged from 6-50% of sample concentration in DOC.

Conservative mixing calculations were calculated using a Model 5011 UIC coulommeter and quantified relative to a sea water certified reference material (CRM). After the addition of 100 μl 20% phosphoric acid, CO2 was stripped with UHP N2, delivered to the analyzer and measured with an RSD of 4% relative to the CRM value (2.2 ± 0.001 ppm). Thirty microliters of 50% phosphoric acid was added to the headspace of the sample vial to allow the DIC to transfer into the headspace as CO2. Samples were shaken vigorously at least once every 15 min for 2 h. Headspace gas from the sample vial was transferred and injected with a 100 μl glass syringe into a Hewlett Packard 5890 GC, where the CO2 was separated isothermally (50 °C) on a Porapak-Q capillary column (30 m, 0.32 mm ID) before isotopic analysis with the Thermo-Finnigan DELTAplus XP IRMS, as described above, with analytical error (1σ) of 1.1‰.

Sulfate and chloride concentrations were determined using a Metrohm 881 Compact Plus ion chromatography (IC) equipped with a Metrosep A Supp 5-250 anion exchange column. Samples from the MFW, MBW, and SGW were diluted to concentrations of 31, 61 and 101, respectively. Peak areas for sulfate and chloride were quantified against equivalently diluted International Association for the Physical Sciences of the Oceans (IAPSO) standard sea water analyzed at the beginning of the run and after every fifth injection. Chloride concentrations (mmol L⁻¹) were converted to mol L⁻¹ and multiplied by 0.001866 to determine salinity (psu). The analytical error for dissolved constituents was ±3.5% of the IAPSO standard sulfate and chloride values.

**Conservative mixing calculations.** Similar to other studies, conservative mixing models were used to differentiate the roles of physical mixing and in situ reactions on the concentrations and isotopic composition of biogeochemically reactive species through the salinity gradient of the subterranean estuary. Internal production yields an excess of the constituent relative to conservative mixing between freshwater and saline end members, while consumption results in depletion. Conservative mixing calculations for methane, DOC, DIC, and sulfate between the shallow low salinity (MFW) and deep high-salinity (SGW) ground-water layers were done using an established approach, adapted for the density-stratified groundwater:

$$ \delta_{\text{MIX}} = \frac{f_{\text{MFW}} \delta_{\text{MFW}} + f_{\text{SGW}} \delta_{\text{SGW}}}{f_{\text{MFW}} + f_{\text{SGW}}} + (1 - f_{\text{MFW}}) \delta_{\text{SGW}} $$

This equation is used to calculate the conservative mixing regime for each layer, and the δ values for the contrasting end members are used in the equation. The δ values are expressed as σC using the equation:

$$ \sigma_{\text{C}} = \frac{\delta_{\text{MIX}} - \delta_{\text{MFW}}}{\delta_{\text{SGW}} - \delta_{\text{MFW}}} $$

where \( f_{\text{MFW}} \) is the fraction of the freshwater end member present in the mixture calculated from the chloride concentration:

$$ f_{\text{MFW}} = \frac{[Cl^-]_{\text{SGW}} - [Cl^-]_{\text{MIX}}}{[Cl^-]_{\text{SGW}} - [Cl^-]_{\text{MFW}}} $$

Environmental lipid biomarkers. Respiratory quinones were extracted using a modified Bligh and Dyer extraction and with DNP-PE-C16:0/DAG (2,4-dinitrophenyl phosphoethanolamine diacylglycerol; Avanti Polar Lipids, Inc., Alabaster, AL) as internal standard and analyzed using a Thermo Q Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source (ESI) connected to an Agilent 1200 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA). Detection of quinones was achieved using positive ion ESI while scanning a m/z range from 100 to 1500. The mass spectrometer was set to a resolving power of 140,000 (FWHM at m/z 200) and 17,500 for MS2 scans. Every analysis was mass calibrated by lock mass correction. The full scan mass resolution setting corresponded to an observed resolution of 75,100 at the m/z 875.5505 of our internal standard, DNP-PE. Ion source and other full scan mass spectrometry parameters were set according to established protocols. MS2 spectra were obtained in data dependent mode. For each M5 full scan, five ions of highest intensity were selected in series using the quadropole for MS3 fragmentation (4 Da isolation window) with a Stepped Normalized Collision Energy of 20, 50, and 80. Analytes were separated using reversed phase HPLC on an Agilent XBridge column (2.1 x 150 mm, 5 μm particle size, Waters Corp., Milford, MA, USA) as described in Collins et al., modified after Hummel et al. Quinones were identified by retention time, as well as accurate molecular mass of proposed sum formulas in full scan mode and tandem MS fragment spectra (Supplementary Fig. 7). Integration of peaks was performed on extracted ion chromatograms using an isolation width of 4 ppm and included the [M + H]+, [M + NH4]+, and [M + Na]+ ions. Quinone abundances were corrected for the relative response of ubiquinone (UQ10) to the different assumed standards (Sigma Aldrich, St. Louis, MO, USA) vs. the DNP-PE standard.

Bulk stable isotopic analysis. Prior to stable carbon isotopic analyses, particulate OM filters, soil, and invertebrate samples were exposed to 10% HCl to remove inorganic carbon, rinsed with ultrapure water, dried, and wrapped in baled (450 °C for 4 h) aluminum cups. Fauna and soil samples were analyzed for 13C and D (H2), and PO4 for 34Si at the University of Alaska Fairbanks (UAF) Stable Isotope facility using established internal protocols. 13C values were measured by Elemental Analyzer Isotope Ratio Mass Spectrometry (EA-IRMS) using a Thermo Fisher Scientific Elemental Analyzer (Flash 2100) combined with Thermo Fisher Scientific Deltaett Plus isotope ratio mass spectrometer and a ConFlo IV interface. 13C values are reported in reference to international isotope standards. The 14 μl m/z 34Si analyses used to quantify the contribution of the silica components for δ34Si values were analyzed on an ANCA-GSL elemental analyzer (Sercon, Crewe, UK) coupled to a Geo20-20 continuous flow IRMS at Iso-Analytical and on a Finnnigan
ThermoQuest thermochemical reactor elemental analyzer (TCEA; Finnigan ThermoQuest, Bremen, Germany) attached via a ConFlo III to a Thermo-Finnigan Delta Plus XP IRMS. Peak areas and isotopic values were determined using Isoplot 3.13 software. δ13C values were reported using the Vienna Pee Dee Belemnite (VPDB) standard.

Contribution of methane-derived carbon to the biomass. A simple two-source mixing model86 was used to calculate relative contributions of methane-derived carbon and the soil-derived carbon (DOC and POC) in the shrimp tissue. The following equation was used for this calculation:

\[ \text{% methane carbon contribution} = \frac{\delta_{\text{shrimp}} - \delta_{\text{soil}}}{\delta_{\text{methane}} - \delta_{\text{soil}}} \times 100 \]  

where \( \delta_{\text{shrimp}} \) is the measured δ13C value of the shrimp, \( \delta_{\text{methane}} \) is the average δ13C value of methane in the MFW, and \( \delta_{\text{soil}} \) is the average δ13C value of DOC in the MFW. This assumption is made in order to simplify the calculation.

Lipid biomarkers from fauna. We performed compound-specific stable carbon isotopic analysis of membrane-bound FA's extracted from tissue of T. philadephus specimens. The examined tissue was removed from the carapace and did not contain gut material. Lipid biomarkers were extracted according to a modification of established methods68. Double bond positions were determined through analysis of their dimethyl-dimethoxymethyl adducts68. Two specimens were selected for this study, one with the lowest (3%) and another with highest (55%) calculated contribution of methane-derived carbon to their biomass. The δ13C values of FA biomarkers and their percentage contributions to the total FA pool extracted from the tissue of the two shrimp specimens are listed in Supplementary Table 5. Reproducibility was monitored by repeated injections and monitoring of internal standards. Reported δ13C values have an analytical error of ±1%.

Phylogenetic analysis and sequence processing. DNA was extracted from 4% of a 47 μm diameter 0.2 μm filter (Pall Soprop) using a PowerViral Environmental RNA/DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer’s recommendations. DNA was eluted into 50 μl of elution buffer and stored at −20 °C. Eluted DNA quality and quantity were evaluated on a NanoDrop ND-100 Spectrophotometer (Thermo Fischer Scientific, USA). The hypervariable V4 region of 16 S rRNA was amplified using modified 515F and 806R primers (Earth Microbiome Project; April 2015). Primers for two-step PCR amplification and library preparation were designed using the TagMeMatrix spreadsheet. Briefly, internal fusion PCR primers were constructed with the priming region for the 16S rRNA locus, a variable length tag (5–8 bp), and a 5' sequence to target for further TruSeq library preparation. The resulting PCR1 products were purified using AMPure XP Beads (Agencourt, Beckman Coulter, USA). PCR2 was used on cleaned PCR1 products to complete TruSeq library fragment and Illumina indexing. Ampure XP cleanup was conducted, libraries were assayed for quality on a BioAnalyzer 2100, quantified on Qubit 2.0 and qPCR was conducted using the New England Biolabs Illumina Library Quantification kit. The library was sequenced on an Illumina MiSeq at the Core Facility for Nucleic Acid Analysis at the University of Alaska Fairbanks. Amplicons derived from sequencing were processed using the DADA2 R-package99. This package implements filtering of low-quality sequences using Q20 individual nucleotide cutoff, merging of paired-end reads, and chimera identification. Reads <150 bp were removed from the analysis and only samples with more than 300 high-quality reads were included in downstream analyses. Taxonomic identification was assigned also in the DADA2 package using RDP90 as the reference database. We determined functional (metabolic) groups by using RDP to search for representative sequences from each of the operational taxonomical unit.

Data availability. Demultiplexed reads were deposited in NCBI Sequence Read Archive (SRA) database under accession number SRP109857. Additional data referenced in this study are tabulated in Supplementary Tables, and available through the USGS Sciencebase Catalog at https://doi.org/10.5066/F7SBD5DW, or on request from the corresponding author (D.B.).

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