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EDITED BY

Enrico Lunghi,
University of L'Aquila, Italy

REVIEWED BY

Benjamin Hutchins,
Texas State University,
United States
Maarten van Hardenbroek,
Newcastle University,
United Kingdom

*CORRESPONDENCE

Amos Frumkin
✉ amos.frumkin@mail.huji.ac.il

[†]These authors have contributed equally to this work and share first authorship

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An isolated chemolithoautotrophic ecosystem deduced from environmental isotopes: Ayyalon cave (Israel)

Amos Frumkin^{1*†}, Ariel D. Chipman^{2,3} and Israel Naaman^{1†}

¹Institute of Earth Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel, ²Department of Ecology, Evolution and Behavior, The Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel, ³The National Natural History Collections, The Hebrew University of Jerusalem, Jerusalem, Israel, ⁴GeoQuEST Research Centre, School of Earth and Environmental, University of Wollongong, Wollongong, NSW, Australia

The stable isotopes composition of chemolithoautotrophic cave ecosystems is known to differ from epigenic caves. Here we show that in addition, dead carbon (devoid of ¹⁴C), is utilized and transferred throughout this ecosystem, rendering it unsuitable for radiocarbon dating. The connectivity of the Ayyalon Cave ecosystem with the surface is studied, along with its sources of energy and carbon, as well as the interconnections between its constituents. We use isotopic evidence to show that its ancient resilient ecosystem is based on an underground food web depending on rich biomass production by chemolithoautotrophic nutrient supplies, detached from surface photosynthesis. Carbon isotopic values indicate that: (1) the microbial biota use bicarbonate from the groundwater (23.34 pMC [% of modern carbon]) rather than the atmospheric CO₂ above the water (71.36 pMC); (2) the depleted ¹⁴C signal is transferred through the entire ecosystem, indicating that the ecosystem is well-adapted and based on the cave biofilm which is in turn based on groundwater-dissolved inorganic carbon. Incubation of Ayyalon biofilm with ¹⁴C-labelled bicarbonate indicates uptake of the radio-labeled bicarbonate by sulfur-oxidizing proteobacteria *Beggiatoa*, suggesting that these sulfur-oxidizing microorganisms use the water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. Organic matter in the cave is much lighter in its stable nitrogen and carbon isotopes compared with respective surface values, as expected in chemolithoautotrophic systems. This evidence may be applicative to subsurface voids of ancient Earth environments and extraterrestrial systems.

KEYWORDS

sulfidic cave, arthropod adaptation, radiocarbon, stable isotopes, environmental isotopes, chemolithoautotrophy, cave ecosystem, biofilm

1. Introduction

Chemoautotrophic micro-organisms use various metabolic strategies to produce biomass by fixing CO₂ at the expense of energy extracted from chemical compounds (Engel, 2007), and support diverse food webs in caves (Culver, 2013). However, most human-accessible caves lack chemoautotrophic production. Chemoautotrophy-based karst ecosystems are often limited not only by energy, but also by the supply of elements such as iron, nitrogen, and sulfur (Sarbu et al., 1996). The dynamics of these elements have a strong influence on the magnitude and location of the energy base of food webs in the cave ecosystems, with zones such as the water-air interface acting as hotspots for productivity. Adaptations in diverse groups of cave-dwelling animals at the physiologic and molecular levels are affected by chemoautotrophy.

Cave chemolithoautotrophic processes were studied mainly since the mid-1990s, in association with studies of deep terrestrial groundwater and deep-sea hydrothermal vents (Stevens and McKinley, 1995). Chemolithoautotrophs have been commonly considered to be inefficient, relative to phototrophs. Therefore, chemolithoautotrophs have been assumed not to be important primary producers. However, under the absence of light and photosynthesis in the subsurface, chemolithoautotrophy can be important, sustaining many ecosystem-level processes (Culver and Pipan, 2019).

Chemolithoautotrophs use compounds present in rocks or groundwater. The energy is obtained by transferring electrons from one chemical compound to another. The electron donors include, for example, reduced sulfur compounds (e.g., hydrogen sulfide, elemental sulfur), molecular hydrogen (H₂), ammonium (NH⁺), reduced iron (Fe²⁺) and manganese (Mn²⁺).

Along with photosynthesis, chemolithoautotrophy has been crucial in the transformation of chemical energy from one reservoir to another during Earth's history and for the incorporation of inorganic carbon into the biospheric organic carbon. Only members of the domains Bacteria and Archaea are capable of chemolithoautotrophy.

In spite of the importance of chemolithoautotrophy, the knowledge of chemosynthetic systems of caves remains limited. Most chemolithoautotrophic caves with well-developed geomicrobiology ecosystems have sulfidic water (Northup et al., 2001; Hose, 2013; Palmer, 2013).

A good example of such well-developed ecosystem with 37 metazoan endemics is Movile Cave (Brad et al., 2021), which has a sulfidic groundwater pond that is close to the surface, but represents a regional aquifer. The aquifer is accessed not only through the cave, but also through boreholes and wells. The diverse hygropetric fauna of Movile is composed mainly of new species which are congeners of genera present in the epigeic surroundings that penetrated the “dry bells” of that cave system possibly in a relatively recent geological past (Sârbu, 2001). The first use of environmental isotopes to study a chemoautotrophic cave system was performed at Movile by Sarbu et al. (1996).

Frasassi Cave in Italy has 12 endemic species, and was studied using stable isotopes by Sarbu et al. (2000). In the Frasassi Cave, evidence for nitrogen fixation was found in the symbiotic bacterial community of *Niphargus* amphipods, *Beggiatoa*-dominated biofilms, and sulfidic sediment (Desai et al., 2013). Dattagupta et al. (2009) showed in the same cave that *Niphargus icus* throughout the cave is colonized by a single phylotype of bacteria in the sulfur-oxidizing clade *Thiothri*.

Cueva de Villa Luz (Hose and Pisarowicz, 1999; Palmer, 2013) has several skylights and a large sulfidic water stream coming out from its subsurface voids. Its connection with the surface fauna is thus larger than the above-mentioned caves, sustaining a smaller number of endemism.

In various caves, including anchialine systems, in Europe, America and Australia, chemoautotrophy has been shown to be either aerobic (oxidation of hydrogen, sulfur, iron, manganese, ammonia or methane) or anaerobic (by methanogenesis, acetogenesis, denitrification or the reduction of sulfate, iron or ammonia) with multiple co-occurring chemolithotrophic metabolic processes in most subsurface systems. In some aquifer systems, ¹³C-depleted biomass indicated the presence of chemoautotrophic pathways through methane-linked carbon cycling, for example in Edwards Aquifer, Texas (Bishop et al., 2014; Hutchins et al., 2016), and Ox Bel Ha, Yucatán peninsula (Pohlman et al., 1997). Based on the prevalence and abundance of ¹³C-depleted lipids, it has been shown that biofilms in lava caves at Lava Beds National Monument, US, are also fueled by *in situ* chemolithoautotrophy (Selensky et al., 2021). Iron-based metabolism is practiced by reducing microorganisms in iron ore caves (Parker et al., 2018.). However, most of the last-mentioned caves lack Metazoa.

Previous studies have used stable environmental isotopes to study chemoautotrophic cave ecosystems. In Movile and Frasassi caves, organisms with a chemolithoautotrophic diet have δ¹³C values ranging from −38 to −46‰ and from −30 to −40‰ respectively (Sarbu et al., 1996, 2000). We hypothesize that such negative values are expected also in other similar chemoautotrophic caves, such as Ayyalon. We therefore test the stable environmental isotopes of Ayyalon, and compare them with their surface counterparts, for testing the dis/connection of the cave ecosystem from above-ground system.

Another hypothesis to be tested here, is that throughout a chemoautotrophic system, the carbon should be mostly ‘dead’, i.e., devoid of ¹⁴C, due to the deep sources of carbon used by the cave ecosystem. This contrasts with epigenic cave systems which rely on carbon produced by photosynthesis using atmospheric CO₂ at the earth surface, i.e., carbon which is rich in ¹⁴C. To test our assumption, we measure the ¹⁴C content throughout the entire ecosystem, in order to differentiate chemoautotrophic components from associated surface phototrophic ones.

We also use the classic tools of carbon and nitrogen stable isotopes to further understand the ecosystem, from basic inorganic materials to highly-endemic arthropods (Por et al., 2013).

2. Ayyalon cave

2.1. Ayyalon hydrogeology

Ayyalon Cave (34.928157°E, 31.910891°N) is located ~20 km east of the Mediterranean coastline. The natural surface is ~100 m asl (above sea level). The Neshar Ramla quarry breached the natural surface during the second half of the 20th century, reaching Ayyalon Cave and associated watertable in 2006. The 2.7 km long Ayyalon Cave is within late Cretaceous (Turonian) massive limestone (Figure 1C). Ayyalon Cave was geologically covered by a ~100 m thick, almost impermeable chalk. Until its truncation by the quarry it had been effectively isolated from the surface by the impervious

rock, which seems to have precluded percolation of water, organic matter and nutrients from photosynthetic surface environments.

A phreatic aquifer is exposed within the cave as a pond, serving as a window into the warm-sulfidic groundwater (Frumkin and Gvirtzman, 2006). The watertable is fluctuating between ~12–24 m asl, thus the water depth of the pond has varied recently between 2 (in 2018) and 9 m (in 2022). The aquifer at Ayyalon vicinity is slightly hydrothermal (also termed ‘Ayyalon anomaly’) with relatively high salinity and H₂S, compared with the regional groundwater (Frumkin and Gvirtzman, 2006). The ‘Ayyalon anomaly’ is a unique part of Israel’s largest aquifer, Yarkon-Taninim (Weinberger et al., 1994), whose regional freshwater is generally cooler, less saline, and devoid of H₂S. Yarkon-Taninim recharge

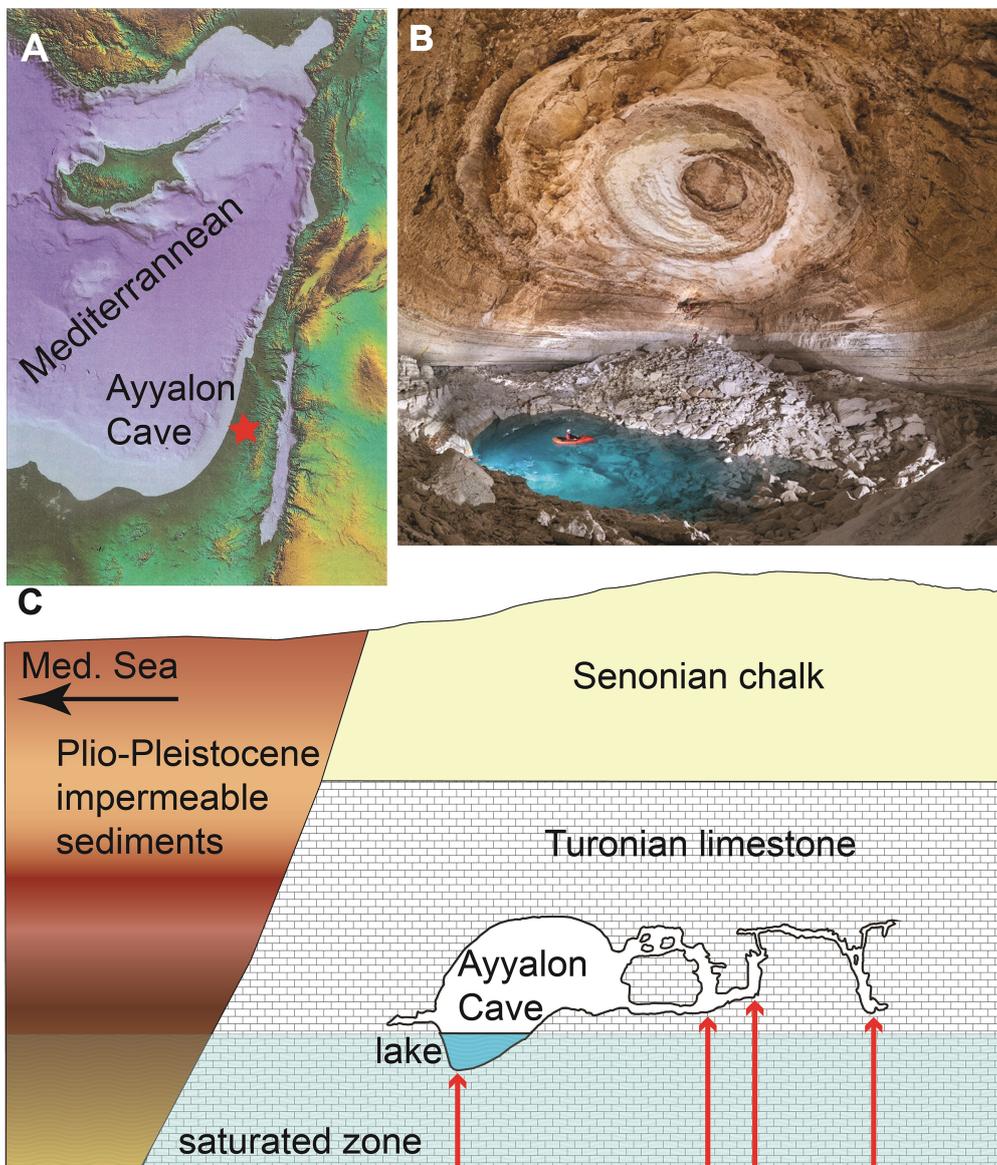


FIGURE 1
 Ayyalon Cave configuration. (A) location; (B) the main chamber and groundwater pond of the cave, acting as the ecosystem focal point (photo: Boaz Langford); (C) schematic geological cross section of the cave and associated hydrogeology. For location of section see blue indicators in Figure 2A.

zone extends along the western slopes of Israel's central highland. The western part of the aquifer is confined by Senonian chalks overlain by younger low-conductivity rocks, dominated by the Pliocene Yafo Formation marls, among other late Cenozoic sediments. Ayyalon Cave is located east of the confinement line.

West of the Neshar Ramla quarry, within the Mediterranean Coastal Plain, the facies of the late Cretaceous carbonate rocks changes laterally, becoming impervious to the west, and together with the overlying Yafo Formation clays, these units block westward groundwater flow (Frumkin et al., 2022). Under these confining and damming conditions within the regional aquifer, the artesian hydrothermal flow mixes with fresh water flowing laterally from the recharge zone along the upper aquifer. The water temperature is up to 30°C at the hydrothermal plumes rising from depth through karstic shafts (Frumkin et al., 2015), while the mean surface air temperature is 20°C. Ayyalon Cave groundwater pond temperature ranges from 28.5 to 29.6°C (Table 1). A thermal-infrared survey performed at the flat bottom of the quarry near the Ayyalon Cave showed groundwater temperatures varying spatially by >3°C, in agreement with direct water temperature measurements in boreholes (Frumkin et al., 2015). The geothermal properties of the groundwater thus include 'hot spots' with temperatures around 2–3°C higher than in the surrounding aquifer. The sulfidic water contains no oxygen below the uppermost layer (Table 1). The spatial distribution of this anomalous water may indicate the possible limits of the aquatic ecosystem, and vice versa. Testing the isotopic features of the ecosystem can thus advance the understanding of water-ecosystem interconnection.

The plan and section of Ayyalon Cave (Figures 1C, 2B), show its affinity to the hypogene environment of rising hydrothermal plumes. The ascending groundwater caused karstic dissolution which formed a hypogenic maze, with intricate network of passages, chambers and rising-water shafts. Some of these have collapsed and advanced upwards, until reaching the surface and forming subaerial sinkholes (Frumkin et al., 2015) which serve as traps for surficial sediments, anthropogenic and zooarchaeological remains (Zaidner et al., 2014; Ullman et al., 2022).

2.2. Ayyalon microbial biota

Ayyalon Cave (and some neighboring smaller caves) is probably the only known deep (> 100 m below surface) sulfidic cave system with endemic metazoan fauna (Figure 1; Por et al., 2013). The cave ecosystem is closely associated with its groundwater pond whose surface is partly covered by microbial mats. Initial microscopic observations indicate that the microbial mass in the water includes filamentous sulfur bacteria with intracellular sulfur granules, spherical sulfur (?) bacteria, amoeboids and other protozoa. Thus the CO₂ fixation may be performed by several microbial taxa. The microbial study of Ayyalon will be published elsewhere.

Metazoan taxa thrive in the water, on the microbial mats, and around the pond, where stranded relic biomats are observed. The Ayyalon Cave ecosystem demonstrates similar conditions and

ecological networks to groundwater in smaller caves and drillings located a few hundred meters away (Frumkin et al., 2022).

TABLE 1 Temperature, oxygen and hydrogen sulfide in Ayyalon Cave groundwater pond at various depths and seasons.

Date (DD-MM-YY)	Depth in water (cm)	Temperature (°C)	O ₂ (mg/l)	H ₂ S (mg/l)
21-05-06	0.1	28.5	3.5	4.7
28-05-06	450	n.d.	0	4.4
14-05-07	10	28.7	1.1	n.d.
19-06-07	10	29	0.11	n.d.
26-07-07	10	29	0.12	n.d.
06-09-07	10	29	0.13	n.d.
25-09-07	10	29.6	0.08	n.d.
01-11-07	10	28.6	0.07	n.d.
31-12-07	10	28.7	0.4	n.d.

The water is thermal (compared to 20°C mean surface temperature), sulfidic, and contains oxygen mainly in the uppermost few mm. n.d. = not determined.

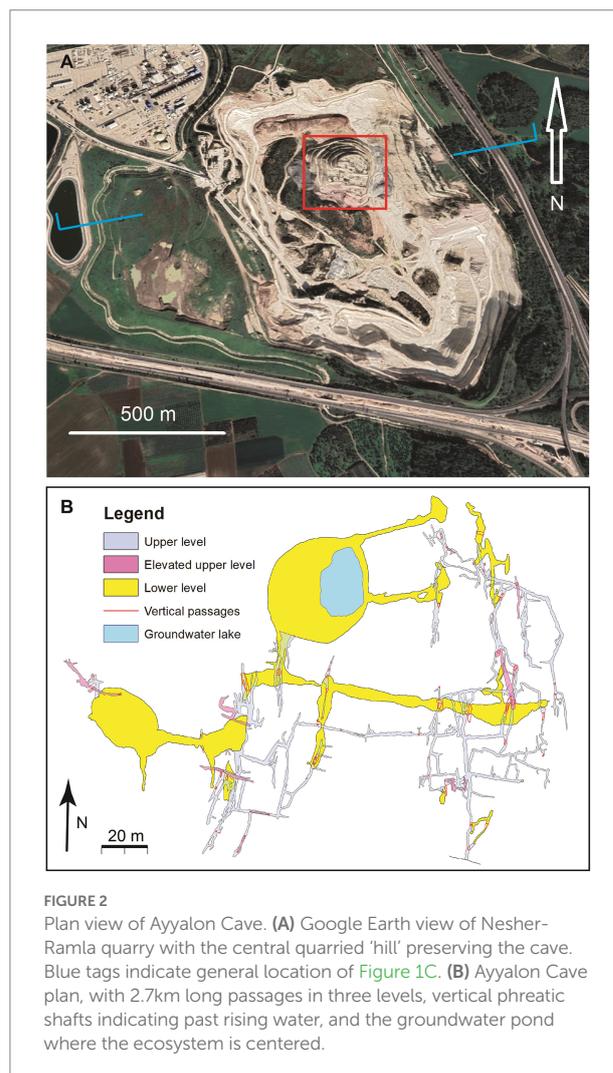


FIGURE 2 Plan view of Ayyalon Cave. (A) Google Earth view of Neshar-Ramla quarry with the central quarried 'hill' preserving the cave. Blue tags indicate general location of Figure 1C. (B) Ayyalon Cave plan, with 2.7km long passages in three levels, vertical phreatic shafts indicating past rising water, and the groundwater pond where the ecosystem is centered.

2.3. Ayyalon fauna

In recent years, most Ayyalon Cave arthropods have been identified and described (Table 2). The species described up to 2022 include the following detritivores and predators: a copepod, a hot-spring shrimp, a crayfish, a pseudoscorpion, a scorpion, and a collembolan. They include endemic species, endemic genera, tribe and possibly endemic family (Levy, 2007; Ćurčić, 2008; Tturnamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012).

Ayyalon Cave crustaceans' stomach content has been studied microscopically in the laboratory in order to identify their food source (Tturnamal, 2008; Wagner, 2012). Phylogenetic studies and molecular comparisons with congeneric relatives were performed on the prawn *Typhlocaris ayyaloni* (Guy-Haim et al., 2018).

Five of the seven arthropod species of the cave are already defined as new and endemic, restricted to the Ayyalon Cave system (Levy, 2007; Ćurčić, 2008; Tturnamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012). The taxonomic position of two additional arthropod species (*Metacyclops subdolos* and *Troglopedetes* sp.) is still unclear, though probably they are also new endemics (Por et al., 2013).

2.3.1. Adaptive traits of Ayyalon fauna

The arthropod fauna of Ayyalon is partly (four species) stygobiont (obligate aquifer-dwelling), and partly (three species) troglobiont (obligate terrestrial cave-dwelling), documented around the pond in a hypogetric environment. The Ayyalon fauna show typical cave adaptations, such as the loss of eyes and pigmentation of the larger species, *Typhlocaris ayyaloni* (Tturnamal, 2008) and *Akrav israchanani* (Levy, 2007).

In addition, some adaptations are more specific to Ayyalon fauna and similar cases. The unusual structure of pedipalp fingertips of *Akrav israchanani* may be an adaptive trait useful for preying on aquatic crustaceans abundant in the groundwater of the cave (Fet et al., 2011).

Laboratory observations on living *Typhlocaris ayyaloni* showed that these prawns do not respond to light, but are very

sensitive to vibrations or to moving of objects in its vicinity (Tturnamal, 2008). The prawns reacted by moving their sensory organs, or by getting into an alert position and retreating when an object was moving in the water as far as 15 cm away. Their stomach contents indicate that they feed directly on microbial mats and also on small crustaceans. The lack of egg-bearing females and very small prawns indicate that the groundwater pond of the cave is used by the prawns as a rich feeding ground, while other cavities of the surrounding aquifer may provide environmental conditions, such as cooler water, suitable for reproduction.

An interesting trait of the cave pond crustaceans is a predominantly upside-down swimming posture of *Tethysbaena ophelicola*, and the occasional back-down swimming at the pool surface of *Typhlocaris ayyaloni*. A similar trait was reported at the sulfurous cave stream in Frasassi, where the amphipod *Niphargus ictus* swims upside-down at the water surface (Flot et al., 2010). Our observation thus supports upside-down swimming to be a general adaptive trait of life for oxygen replenishing in a thin (mm thick) layer of oxygenated water at the top of the groundwater. The life of the sulfidophilic *Tethysbaena* under microaerobic and anaerobic conditions relates to the biochemical and physiological adaptations to anoxia of the whole crustacean order Thermosbaenacea (Por et al., 2013). *Tethysbaena* has developed survival mechanisms to anaerobism, resistance to hydrogen sulfide poisoning, to elevated water temperatures as well as to fluctuating dissolved mineral content. Sulfide is most probably detoxified by intestinal bacteria (Por, 2014).

3. Materials and methods

3.1. Cave measurements and sampling

A geologic-geomorphic field survey of Ayyalon Cave was conducted. The cave was surveyed using the following instruments: Leika™ 'Disto' laser range-meter, inclinometer and compass (Dasher, 2011); plans and cross-sections were drawn to the scale of 1:200 using LimeLight™ cave mapping software. The cave map is presented in Figure 2B. In order to avoid contamination of the cave, the equipment was cleaned and disinfected prior to field studies in the cave.

Aquatic arthropod samples were collected using 60 and 200 μm nets. Terrestrial arthropods were collected by traps and tweezers. Ultraviolet flashlights were used for locating scorpion remains. Inorganic and organic samples collected within the cave were transferred into a cooling reservoir (~5°C) until submission to the relevant laboratory (see below), which occurred within 18 h after sampling. Similar methods were used to collect aquatic and terrestrial arthropods from the surface outside the cave, for isotopic comparison. Three surface winter ponds were sampled at Innaba, (31.9080N 34.9590E). A pond, ~500 meters south-west of the quarry was not used, due to possible contamination from a nearby sewage treatment plant.

TABLE 2 Arthropod species at Ayyalon Cave.

Species	References
<i>Typhlocaris ayyaloni</i> (Tturnamal, 2008; Crustacea: Decapoda)	Tturnamal (2008)
<i>Metacyclops longimaxillis</i> (Defaye and Por, 2010; Crustacea: Copepoda)	Defaye and Por (2010)
<i>Metacyclops subdolos</i> Kiefer, 1938 auctorum (Crustacea: Copepoda)	Por et al. (2013)
<i>Tethysbaena ophelicola</i> Wagner, 2012 (Crustacea: Thermosbaenacea)	Wagner (2012)
<i>Akrav israchanani</i> Levy, 2007 (Arachnida: Scorpiones)	Levy (2007)
<i>Ayyalonia dimentmani</i> Ćurčić, 2008 (Pseudoscorpiones: Chthoniidae)	Ćurčić (2008)
<i>Troglopedetes</i> sp. (Collembola: Paronellidae)	Por et al. (2013)

Terrestrial arthropods were collected from the vicinity of the ponds.

Biological samples were separated at the Hebrew University and sent for morphological taxonomic identification by specialists of each arthropod group (Levy, 2007; Ćurčić, 2008; Tsumamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012) and for isotopic analysis.

3.2. Carbon and nitrogen isotope analysis

We measured radiocarbon (coupled with $\delta^{13}\text{C}$) isotopic compositions which serve as direct indicators for the connectivity, carbon source of the ecosystem, and its food webs adaptations (Rundel et al., 2012). Each organic sample for ^{14}C was pre-treated at the Weizmann Institute of Science. Any foreign carbon material which is not related to the sample and represents therefore a possible source of contamination was visually removed. Using Fourier Transform Infrared Spectroscopy (FTIR), the material was analyzed before and after pre-treatment to check the possible presence and removal of contaminants. Contaminants such as gypsum were removed by ultrasonication in distilled water. The material was then oxidized to CO_2 and then graphitized for Accelerator Mass Spectrometry analysis and determination of radiocarbon content. A fraction of the CO_2 was used for $\delta^{13}\text{C}$ analysis. The AMS radiocarbon measurements were carried out on a NEC 1.5SDH-1 0.5MV Pelletron with a 40-sample MC-SNICS ion source. The accuracy of this system is higher than 0.4% and the machine background is lower than 0.03 pMC. Specific accuracy is noted per sample in Table 3. $\delta^{13}\text{C}$ is reported by ‰ VPDB (relative to Peedee belemnite) with an accuracy of $\pm 0.15\text{‰}$. For additional data on preparation and ^{14}C Measurement see Boaretto et al. (2009). Atmospheric isotopic values are given after Hua et al. (2021).

Organic samples were analyzed for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotopes at the Institute of Earth Sciences, The Hebrew University of Jerusalem. Samples were dried in an oven at 50°C for 24 h. Calcite was removed from biofilms by adding 10% HCl. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C/N ratios were measured by elemental analyzer / continuous flow isotope ratio mass spectrometry (EA/CF-IRMS). The system included: FlashEA 1,112 analyzer coupled with Thermo Finnigan Delta Plus XL isotope ratio mass spectrometer. The results are reported for $\delta^{13}\text{C}$ ‰ VPDB (IAEA standard, value of zero 0.01123720) with an error up to 0.15‰ , and for $\delta^{15}\text{N}$ (‰ relative to air, IAEA-N-1 standard +0.4) with an error up to 0.15‰ . Error in %C / %N ratio is up to 2%.

Inorganic samples were collected in clean glass bottles from cave pond water, mud at the pond bottom, limestone bedrock at the cave chamber wall, air above the water, and air in an upper cave passage. For dissolved inorganic $\delta^{13}\text{C}$ in water, water was filtered in the field by $0.45\ \mu\text{m}$ filter into a 100 ml glass bottle pre-treated with HgCl. An aliquot of sample was acidified with 5% H_3PO_4 in a gas stripping reactor and $\delta^{13}\text{C}$ was measured for the liberated CO_2 with

TABLE 3 Carbon isotopes in organic matter of Ayyalon Cave and in possible sources of carbon.

Studied material	pMC	pMC E \pm	$\delta^{13}\text{C}$ (‰ VPDB)
1 Limestone bedrock	0.00	0.20	-0.21
2 Atmosphere	104.00	0.27	-8.0
3 Air in high passage	97.93	0.43	-12.2
4 Air above water	71.36	0.33	-14.3
5 Mud (pond bottom)	0.72	0.07	-1.5
6 Water (inorganic C)	23.34	0.26	-12.2
7 Live biofilm	29.65	0.20	-41.8
8 Relic, hanging biofilm	25.28	0.27	-43.1
9 <i>Typhlocaris ayyaloni</i>	25.85	0.19	-33.5
10 <i>Akrav israchanani</i>	24.60	0.19	-32.8

pMC E \pm is the error of ^{14}C ; $\delta^{13}\text{C}$ is given in ‰ VPDB, with error of 0.15‰ . 1. Turonian limestone bedrock; 2. Atmosphere above the cave; 3. CO_2 in an upper passage of Ayyalon Cave; 4. CO_2 of air above Ayyalon Cave pond, within the cave; 5. Inorganic carbon of carbonate mud at Ayyalon pond bottom; 6. Dissolved inorganic carbon in Ayyalon pond water; 7. Biofilm at Ayyalon water surface; 8. Relic biofilm from previous Ayyalon groundwater surface, hanging today on walls above the water, 26 m asl. 9. *Typhlocaris ayyaloni*, Ayyalon Cave pond; 10. Exoskeleton chitin of dead *Akrav israchanani* specimen from Ayyalon Cave.

a Thermo Scientific IRMS system with an accuracy of 0.1‰ VPDB. For solid-phase calcite samples, $\delta^{13}\text{C}$ was measured by gas bench continuous-flow isotope ratio mass spectrometry (CF-IRMS) system including GasBench III connected to Delta Plus XL mass spectrometer with an error up to 0.1‰ (VPDB).

To test whether the difference between the cave and surface samples in Ayalon differ significantly in their isotope values we applied two complementary multivariate approaches, namely a parametric MANOVA (Clarke, 1993, using SPSS statistical package) and a non-parametric ANOSIM (Clarke, 1993) modification (using MATLAB software). For the MANOVA analysis we used Pillai's trace parameter defined between zero and one where zero indicates no difference between the groups and a value closer to one indicates a more significant difference. In ANOSIM the R statistic is used, where R is expected to equal zero when groups do not differ and to be greater than zero when they do. The ANOSIM analysis was performed by comparing the observed R_o statistic to 10,000 random values of the statistic, R_e , expected under the null hypothesis. The values of the statistic were calculated using distance matrix based on Euclidean distances. Random statistic values were obtained by randomly shuffling the observations groupings.

Table 4 shows the stable isotopes values, in which each number is averaged between two measurements.

3.3. Water measurement and analysis

Water temperature and oxygen concentration were measured in 10 cm depth of the cave pond using a Solinst Levellogger DIVER 3001. Dissolved oxygen was measured there with a WTW OXI 330 hand-held meter. Temperature, oxygen and hydrogen

sulfide of the groundwater pond at various depths and seasons are given in [Table 1](#).

Water from Ayyalon Cave pond was sampled directly from the water surface using a peristaltic pump from 10 to 450 cm depth. Each sample for H₂S concentration was collected in three 1.5 liter bottles. Sulfide was precipitated in the field as ZnS by adding 1–2 ml 1N Zn-acetate to 1 liter sampling bottles prior to sampling. The samples were analyzed at the Geological Survey of Israel. Excess water was removed in the lab, and the remaining deposit and water were reacted in a beaker with KI, HCl, and I₂, the latter in excess, which was then titrated using S₂O₃. For additional details see [Gavrieli et al. \(2001\)](#).

3.4. Primary productivity estimation

The ¹⁴C incubation method was used to estimate the uptake of dissolved inorganic carbon (DIC) by microbial mats from Ayyalon groundwater, as was done earlier for other chemoautotrophic caves ([Porter et al., 2009](#)). The method is based on the fact that the biological uptake of ¹⁴C-labeled DIC is proportional to the biological uptake of ¹²C-DIC. The incubation was performed in Aharon Oren's laboratory at the Hebrew University according to [Oren \(2009\)](#).

4. Results and discussion

4.1. Radiocarbon

During the study, the atmospheric ¹⁴C content of CO₂ outside the cave, in the upper cave levels, and above the groundwater pond were 104.0, 97.93, and 71.36 pMC, respectively, ([Table 3](#); [Figure 3A](#)). In contrast, inorganic carbon compounds associated with the cave water and bedrock were highly depleted in ¹⁴C: The Cretaceous bedrock, mud (at the bottom of the pond), and inorganic dissolved carbon of the groundwater contained 0.0, 0.72, and 23.34 pMC, respectively. The mud value represents the dominance of old, 'dead' carbon in the inorganic mud, while the dissolved carbon includes a signature of some recent groundwater flowing laterally from the east.

The measured ¹⁴C content in the cave organisms was 29.65, 25.28, 25.85, and 24.6 pMC for the living biofilm, stranded relic biofilm (hanging above the pond, associated with previous high watertables), living aquatic prawn *Typhlocaris ayyaloni* and exoskeletons of the scorpion *Akrav israchanani* respectively, representing the highest trophic level of the aquatic and terrestrial Ayyalon ecosystem ([Table 3](#); [Figure 3A](#)).

The intake of dissolved carbon from groundwater is further demonstrated by incubation of the biofilm with ¹⁴C-labeled bicarbonate ([Figure 4](#)). Ayyalon microbiota, sampled from the sulfidic cave pond, was incubated with ¹⁴C-labelled bicarbonate. Increase of radioactivity of the microbial lipids during incubation indicates uptake of the radiolabeled bicarbonate by *Beggiatoa*,

suggesting that these sulfur-oxidizing microorganisms use water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. The experiment was performed with and without adding sulfide, showing similar results. *Beggiatoa* sp. of the microbial mat was sampled from the top of the water column (upper lines in [Figure 4](#)), and showed active CO₂ fixation. Lower lines ([Figure 4](#)) indicate no carbon fixation by other bacteria sampled from the water body.

4.2. Stable isotopes

Stable isotopes (δ¹³C and δ¹⁵N) and C/N ratios of the biotic system were measured for terrestrial and aquatic organisms inside the cave, and for comparison, from similar taxonomic groups on the regional surface above the cave ([Table 4](#); [Figures 3, 5](#)). The measurements inside the cave included the entire range of organisms, from biofilms through the living aquatic prawn *Typhlocaris ayyaloni* and exoskeletons of the scorpion *Akrav israchanani*.

Cave vs. surface isotopic values fall in distinctively different fields: Organic matter in the cave is lighter isotopically than surface values (for isotopic ranges, see [Figure 5](#)). Higher trophic levels generally fall to the right of their food source.

4.3. Inference from isotopic values

Radiocarbon is used here to decipher the ecosystem's connectivity with the surface. Recently recharged groundwater, arriving from rainfall, carry an atmospheric signature. In the Judean hills, the radiocarbon content in the groundwater bicarbonate is ~80 pMC, representing mainly a combination of biogenic soil CO₂ and smaller portion of old bedrock carbonate. At Ayyalon, our measured ¹⁴C values of air CO₂ outside the cave, in the upper cave levels, and above the groundwater pond were 104.0, 97.93, and 71.36 pMC, respectively, ([Table 3](#); [Figure 3A](#)).

The negligible ¹⁴C content of the cave mud ([Table 3](#); [Figure 3A](#)) indicates the dominance of decomposed bedrock source. The 71.36 pMC of air above the water indicates the mixing of atmospheric CO₂ from the upper cave levels with depleted carbon from the water. The 23.34 pMC of the cave groundwater inorganic carbon is highly depleted in ¹⁴C, in clear contrast with both the young atmosphere above the cave and the regional young groundwater. This indicates that the cave groundwater is mostly old, rising from the depth, after a long-term ¹⁴C decay and interaction with carbonate bedrock, in contrast with regional young groundwater, whose ¹⁴C content was ~80 pMC ([Kroitoru et al., 1992](#)). This verifies the hydrogeologic data, suggesting that Ayyalon groundwater comprises hydrothermal plumes rising from depth ([Frumkin and Gvirtzman, 2006](#); [Frumkin et al., 2015](#)).

Cave water oxygen is limited to 0.02–3.5 mg/l, within the uppermost mm-thick groundwater layer, while deeper water demonstrates anaerobic conditions. This redox interface and

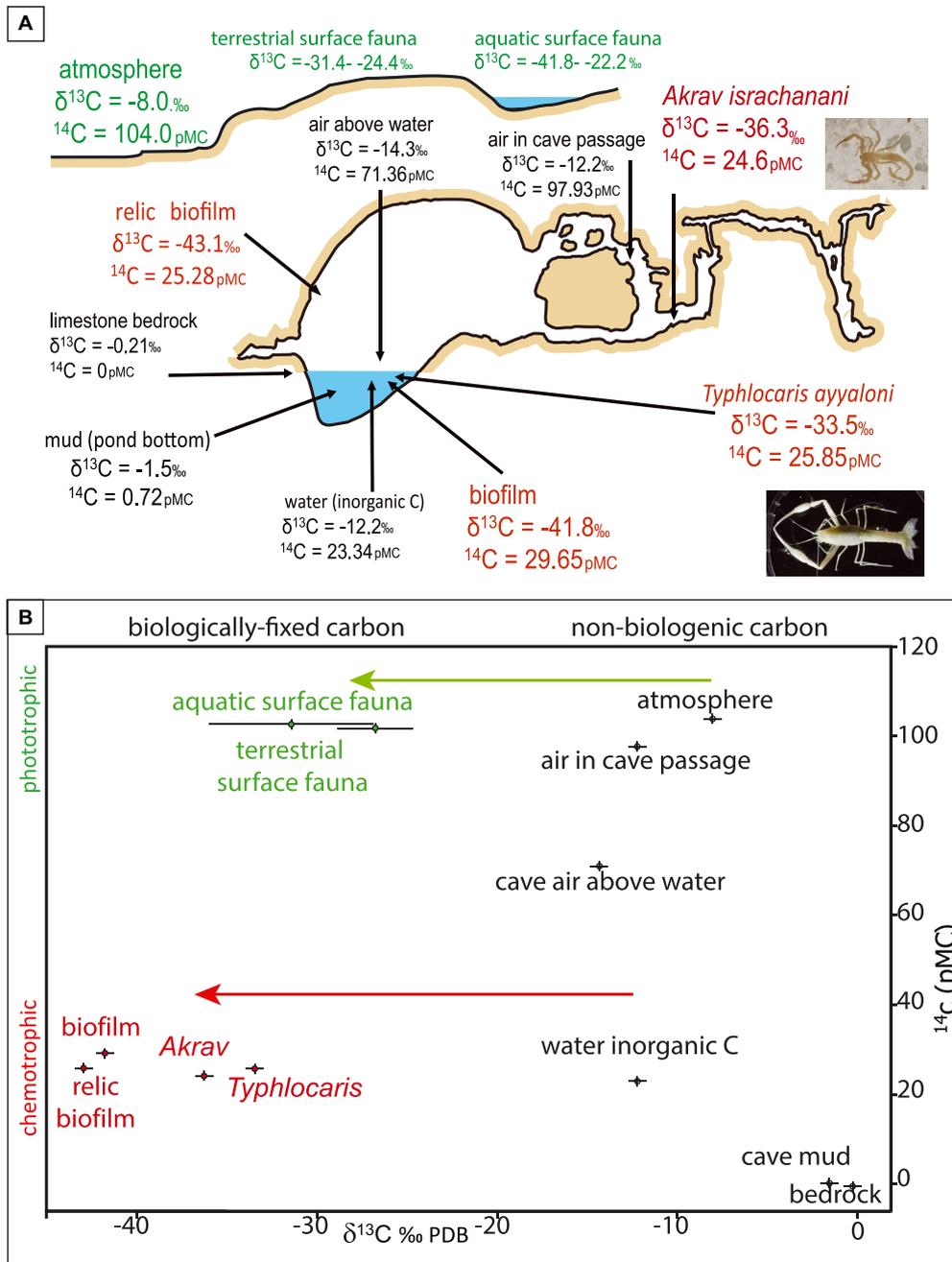
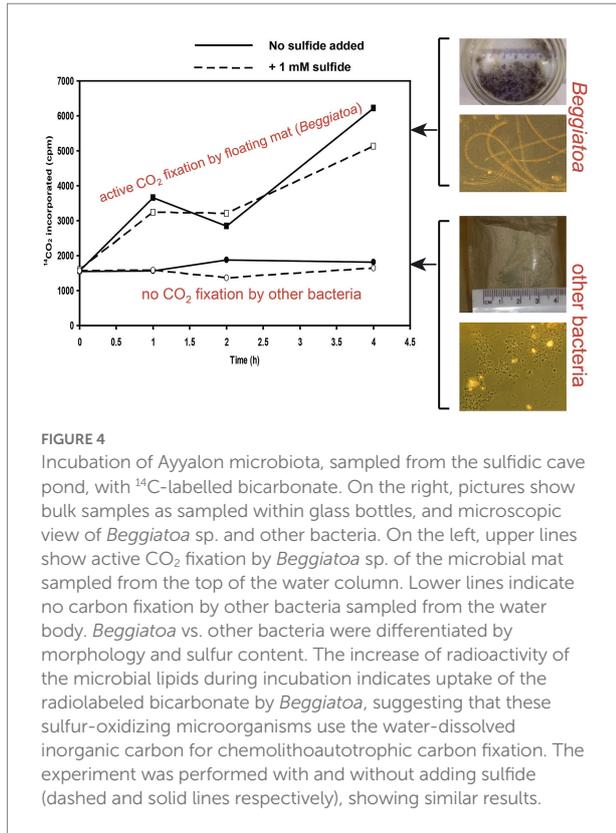


FIGURE 3

Carbon isotopes of the Ayyalon cave and surrounding surface, showing the isotopic difference between the surface phototrophic (green) and cave chemotrophic (red) systems. ^{14}C of surface fauna was assumed to replicate the atmospheric value. (A) cartoon indicating the measured values of the samples and their general location. (B) the relation between ^{14}C and $\delta^{13}\text{C}$ of the system's components. Green arrow represents $\sim 20\%$ fractionation associated with photosynthesis by C_3 plants, subsequently consumed by surface fauna (shown). Red arrow represents fractionation associated with chemoautotrophic sulfur-oxidizing bacteria (Ruby et al., 1987). Individual samples with 1σ laboratory error are shown, except for surface fauna, whose gross diversity is represented by the average values of 15 aquatic species and 11 terrestrial species, with 1σ standard deviation of each group (further details in Tables 3, 4).

associated environment, although poor in oxygen, allows for the rich metazoan life within the groundwater. Specific adaptations to these environmental conditions are discussed above (Section 2.3).

The Ayyalon ^{14}C results (Table 3; Figure 3) indicate that: (1) the microbial biota (29.65 pMC) use mainly bicarbonate from the groundwater (23.34 pMC) with a smaller fraction of atmospheric CO_2 from above the water (71.36 pMC); and (2) the depleted ^{14}C



signal passes through the entire ecosystem, indicating that the ecosystem is totally based on the biofilm which is in turn based on groundwater-dissolved inorganic carbon.

The increase of radioactivity of the microbial lipids during incubation of the biofilm with ^{14}C -labeled bicarbonate (Figure 4) indicates uptake of the radio-labeled bicarbonate from groundwater, suggesting that the sulfur-oxidizing proteobacteria *Beggiatoa* use the water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. This is corroborated by the observation that the cave lacks any organic or inorganic material originating from the surface, due to the cave's isolation.

Further insight into Ayyalon ecosystem and its adaptation is obtained from stable isotopes ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of the biotic system (Table 4; Figure 5). Most organisms with a chemolithoautotrophic diet have quite negative $\delta^{13}\text{C}$ values (e.g., -38 to -46‰ in Movile; -30 to -40‰ in Frasassi; Sarbu et al., 1996, 2000). This is mainly due to discrimination against ^{13}C , associated with chemolithoautotrophic assimilation of inorganic carbon. Methane-driven chemoautotrophic carbon-fueled ecosystems can have $\delta^{13}\text{C}$ values from -30 to -90‰ (Grey, 2016). While $\delta^{13}\text{C}$ of dissolved inorganic carbon of Ayyalon Cave water is -12.2‰ VPDB, the $\delta^{13}\text{C}$ of the biofilm is -41.8 and -43.1‰ (for live and stranded relic samples respectively, Figure 3), slightly larger than expected for fractionation of stable carbon isotopes during chemolithoautotrophic growth of sulfur-oxidizing bacteria between the inorganic carbon of the water and the chemoautotrophic microbiome of the water (Degens, 1969; Ruby et al., 1987; Galimov,

2006). The reasons causing the difference should be further investigated. The depletion is larger than observed in most photosynthetically-derived organic material. The largest degree of fractionation of carbon isotopes occurs during the initial steps of reduction of inorganic carbon (i.e., carbon dioxide and bicarbonate) and results in a decreased $^{13}\text{C}/^{12}\text{C}$ isotope ratio in the organic product relative to that of the source carbon. Subsequent utilization of the organic carbon through trophic levels of the food chain leads to small but detectable increases in the $^{13}\text{C}/^{12}\text{C}$ isotopic ratio (Ruby et al., 1987; Galimov, 2006). Alternatively, isotopically light carbon may rise from the depths as ^{13}C -depleted methane, followed by oxidation to CO_2 and bicarbonate near the water-table, being then metabolized by the biofilm into organic matter (Grey, 2016).

Mean $\delta^{13}\text{C}$ of aquatic and terrestrial species in the cave is -35.2 ± 1.5 and $-32.7 \pm 0.1\text{‰}$ respectively, both much lower than surface samples averaging -28.65 and $-26.8 \pm 2.1\text{‰}$ respectively, which are dominated by the lower carbon fractionation of C_3 photosynthetic vegetation (Figure 3). Within the cave ecosystem, the higher trophic levels show variable isotopic shift relative to their diet (Figure 3B), with a positive shift in both aquatic (-33.0 to -36.3‰ , Table 4) and terrestrial fauna (-32.6 to -33.5‰). This is in agreement with the expected isotopic shifts between diets and consumers (Galimov, 2006).

Similarly, the mean $\delta^{15}\text{N}$ of Ayyalon aquatic and terrestrial organic samples is $-1.0 \pm 1.7\text{‰}$ and $-6.5 \pm 3.3\text{‰}$ respectively, both lower than surface samples, whose average measured values are 6.56 ± 2.9 and $3.4 \pm 3.6\text{‰}$ respectively (Figure 5A). The negative trophic shift of N may be indicative of fluid-feeding consumers. McCutchan et al. (2003) showed that estimates of trophic shift for nitrogen differ systematically between fluid-feeding consumers and other consumers.

Both multivariate tests used here (MANOVA and ANOSIM; Clarke, 1993) indicate a highly significant effect of habitat (cave vs. surface) on the isotopic values of the samples. Pillai's trace value was close to unity (0.995) with $F_{(2,22)} = 2,402$ and $\text{sig.} < 0.0001$. The ANOSIM analysis produced similar results with the observed statistic, $R_o = 0.0267$, being significantly greater than zero – $\text{sig.} < 0.0001$ (Figure 6).

The combined evidence suggests that the sulfur-oxidizing microbiota obtain the necessary carbon for metabolic processes from bicarbonate in the groundwater, as well as nitrogen from nitrogen compounds in the water. The biofilm covering the cave water is thus the primary producer for the entire food chain in the cave, both inside and outside the water.

The higher trophic levels in Ayyalon are evident also by direct observations: The small crustacean *Tethysbaena ophelicola* has only biofilm cells in its stomach, and is itself found in the stomachs of the other crustaceans (Wagner, 2012), indicating that this species is a primary consumer in the Ayyalon ecosystem. The largest crustacean of Ayyalon, *Typhlocaris ayyaloni*, feeds also on biofilms in addition to predation of other crustaceans (Tturnamal, 2008). This is in agreement with the isotopic evidence presented above.

The observed isotopic depletion relative to surface values at Ayyalon are in agreement with measurements in other sulfidic

TABLE 4 Carbon and nitrogen stable isotope values of whole-body biogenic materials in Ayyalon Cave and associated surface near the cave.

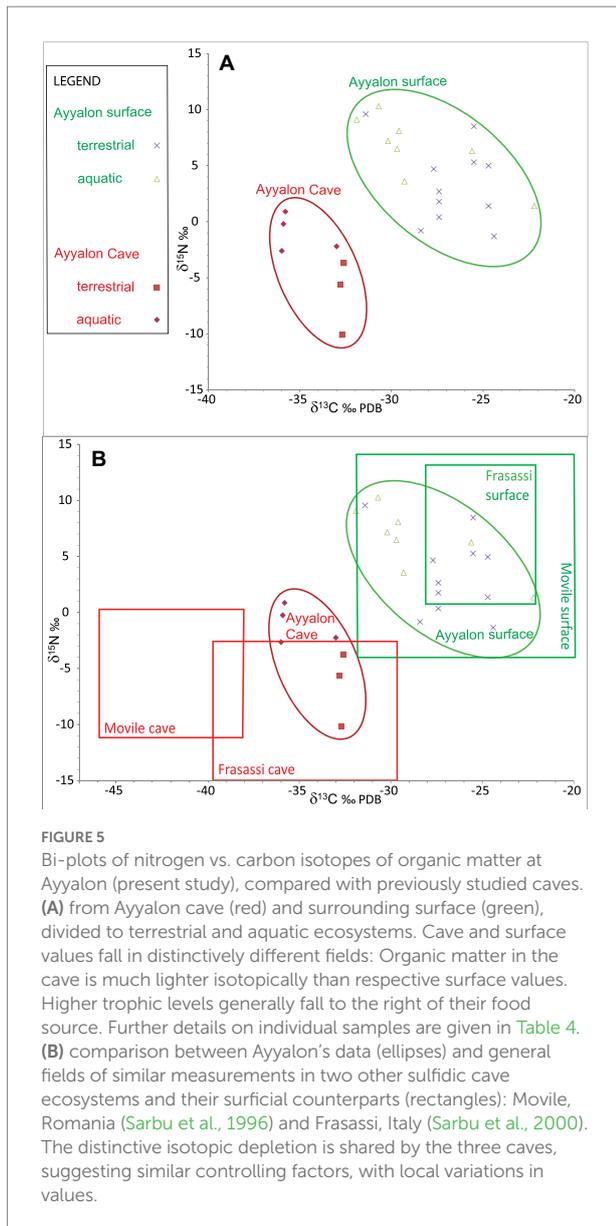
Sample number	Source	$\delta^{13}\text{C}$ ‰ VPDB	$\delta^{15}\text{N}$ ‰ (V. air N_2)	C:N	Taxon
B1	Ayyalon aquatic	-35.8	0.9	5.5	biofilm
A10	Ayyalon aquatic	-33.0	-2.2	4.2	<i>Cyclopoidae</i>
A8	Ayyalon aquatic	-36.0	-2.6	4.7	<i>Tethysbaena</i>
B5	Ayyalon aquatic	-35.9	-0.2	5.1	<i>Typhlocaris ayyaloni</i>
Aquatic average	Ayyalon aquatic	-35.2	-1.0	4.9	
Std. deviation	Ayyalon aquatic	1.5	1.7	0.6	
B7	Ayyalon terrestrial	-32.7	-10.1	5	<i>Collembola</i>
B6	Ayyalon terrestrial	-32.6	-3.7	4.4	<i>Ayyalonia dimentmani</i>
A12	Ayyalon terrestrial	-32.8	-5.6	5.1	<i>Akrav israchanani</i>
Terrestrial average	Ayyalon terrestrial	-32.7	-6.5	4.8	
Std. deviation	Ayyalon terrestrial	0.1	3.3	0.4	
A3	Surface aquatic	-22.2	1.4	9.8	<i>Ostracoda</i>
D10	Surface aquatic	-29.6	8.1	4.5	<i>Corixidae</i>
C5	Surface aquatic	-29.7	6.5	4.9	<i>Coleoptera</i>
C6	Surface aquatic	-31.9	9.1	5.7	<i>Cyclopoidae</i>
C8	Surface aquatic	-30.7	10.3	4.4	<i>Chironomidae</i>
C9	Surface aquatic	-25.6	6.3	4.2	<i>Coleoptera</i>
C10	Surface aquatic	-30.2	7.2	3.6	<i>Zygoptera</i>
C11	Surface aquatic	-29.3	3.6	3.7	<i>Hyla</i> (tadpole)
Average aquatic	Surface aquatic	-28.65	6.56	5.1	
Std. deviation	Surface aquatic	3.17	2.9	2.01	
B9	Surface terrestrial	-28.4	-0.8	3.4	<i>Acrididae</i>
D3	Surface terrestrial	-27.4	0.4	3.3	<i>Grylloidea</i>
B4	Surface terrestrial	-31.4	9.6	4.3	<i>Gryllotalpidae</i>
A4	Surface terrestrial	-27.7	4.7	4.7	<i>Aranae</i>
B10	Surface terrestrial	-24.4	-1.3	7	<i>Chilopoda</i>
B11	Surface terrestrial	-25.5	5.3	3.7	<i>Scorpiones</i>
C1	Surface terrestrial	-27.4	2.7	4.3	<i>Aranae</i>
D1	Surface terrestrial	-24.7	1.4	6	<i>Coleoptera</i>
D7	Surface terrestrial	-27.4	1.8	6	<i>Limacus</i>
D8	Surface terrestrial	-25.5	8.5	4.2	<i>Aranae</i>
D9	Surface terrestrial	-24.7	5	5.1	<i>Coleoptera</i>
Average terrestrial	Surface terrestrial	-26.8	3.4	4.7	
Std. deviation	Surface terrestrial	2.1	3.6	1.2	

Exceptions are exoskeleton chitin of dead *Akrav israchanani* specimen from Ayyalon Cave (sample A12) which was compared with exoskeleton chitin of a living scorpion specimen from the surface (sample B11). The presented results are averages of two samples per each taxon with an error up to 0.1‰ (VPDB).

caves ecosystems, such as Movile (Sarbu et al., 1996), and Frassasi (Sarbu et al., 2000; Figure 5). All these caves show compatible isotopic differences between the surface ecosystem and the subsurface sulfidic chemolithoautotrophy counterpart (Figure 5B). These offsets of $\delta^{13}\text{C}$ values of organisms inside the cave vs. outside are -18 to -14‰ at Movile, -8 to -12‰ at Frassasi, and -4 to -11‰ at Ayyalon Cave. The offsets of $\delta^{15}\text{N}$ are -7 to -14‰ at Movile, -16 to -17‰ at Frassasi, and -9 to -10‰ at Ayyalon

Cave. This indicates that the biofilms in all these sulfidic environments make similar use of carbon and nitrogen from deep sources rather than from the atmosphere.

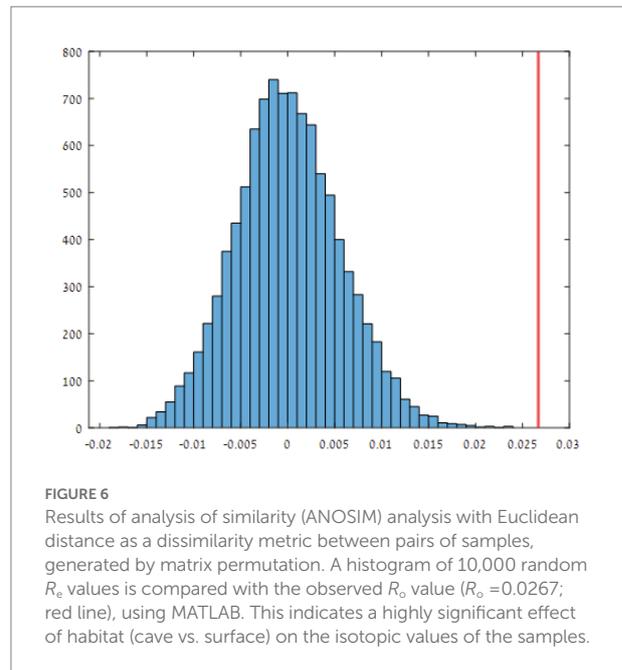
However, there are basic differences between the various cave systems. Frassasi Cave has a better connection with the surface, including a natural entrance with open outflow of its subsurface sulfidic stream (Sarbu et al., 2000). The more secluded Movile Cave has a groundwater pond, is located under 20 m-thick caprock



and soil, and is a rich, ecosystem with many endemic species (Sarbu et al., 1996; Brad et al., 2021). With several faunistic pieces of evidence indicating intermittent connection with the surface, Movile is believed to have been colonized by various species over a much shorter period than Ayyalon, including the late Quaternary. Other known chemolithoautotrophic caves, including anchialine ones, seem to be less isolated than Ayyalon, and commonly have less developed endemic ecosystems (Pohlman et al., 1997; Pohlman, 2011; Hutchins et al., 2016; Engel, 2019).

4.4. Endemism

The taxa of Ayyalon include endemic faunal tribes and/or families (the scorpion *Akrav israchanani* and the pseudoscorpion *Ayyalonia dimentmani*) evolving independently since the Miocene



(Levy, 2007; Ćurčić, 2008; Fet et al., 2011). The high-rank endemism and the deep, well-covered setting, suggest that Ayyalon is one of the most secluded metazoan cave ecosystems known today, whose biota may have been virtually disconnected from other ecosystems for millions of years (Ćurčić, 2008; Fet, 2017; Guy-Haim et al., 2018; Frumkin et al., 2022). However, the stable isotopes data from Movile and Frasassi caves indicate that they are more depleted than their associated surface ecosystems compared with the Ayyalon system (Figure 5B). This might be associated with different emissions of deep-sourced gases, such as methane, in each cave.

The high endemism, long-term (millions of years) resilience (Frumkin et al., 2022), and *in situ* evolution of the Ayyalon fauna suggest that similar chemolithoautotrophic ecosystems could be self-sustained for long (geologic scale) periods, allowing significant behavioral and physiological evolution, associated with adaptations to subsurface secluded conditions (section 2.3.1).

5. Conclusion

This study shows that under the lack of epigenic sources of carbon and energy, the Ayyalon Cave ecosystem has adapted by using internal carbon and energy sources. This is demonstrated, for the first time, by the low ^{14}C content of the ecosystem, indicating the uptake of inorganic carbon from old groundwater. The dead carbon used by microbial mats in the cave is recognized throughout all the trophic levels, demonstrating that in such ecosystems dead organisms cannot be dated by ^{14}C .

The ^{14}C evidence is corroborated by the stable isotopic values of the ecosystem, which show a major depletion of C and N isotopes throughout the cave ecosystem. The low values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ indicate underground chemolithoautotrophic production,

rather than allochthonous import from epigeal systems. The high endemism of the ecosystem shows that it has been detached from surface terrestrial and marine environments for a long period, amounting to millions of years. Our initial assumptions of the isotopic values of such an ecosystem thus prove to be true.

Our results also demonstrate the sustainability and adaptation of a deep isolated subsurface ecosystem. This may suggest the possibility of long-lived cave ecosystems early in the evolution of terrestrial life, and may shed light on potential extraterrestrial ecosystems. The subsurface of Mars and other planets is protected from the damaging effects of short-wave UV radiation and the oxidizing nature of the surface, thus providing a promising habitat for life (Chyba and Hand, 2005; Kaltenegger, 2017). In particular, caves and other subsurface environments may be habitable where sufficient geothermal heating permits the existence of liquid water. Such features make subsurface environments on exoplanets seem more plausible for life than their surface counterparts.

The secluded Ayyalon system is in great danger since it was breached by the quarry. The Nature Reserves Authority of Israel is urged to take active measures of conservation in order to avoid disturbance to the fragile system, and keep it as secluded as possible.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

We declare that the research have been conducted in accordance with Frontiers guidelines on study ethics. The study is exempt from ethics approval, due to the following reasons: No vertebrates have been involved in the study. No research on human subjects have been conducted.

Author contributions

AF coordinated the study and wrote the paper. AC ensured biological validity and edited the text. IN performed the field work

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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