

# Chemolithoautotrophic Organic Matter Contributions to Subterranean Food Webs Dominated by Filter-feeders

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## Abstract

### Introduction

Most groundwater and cave ecosystems depend on an influx of allochthonous, surface-derived organic matter sourced by diffuse flow through overlying rock and soil or by localized flow from the surface into sinkholes or entrances. The amount of organic matter entering the subsurface is usually low, resulting in oligotrophic conditions and food scarcity that affect community members' dispersal and colonization patterns. In situ, chemolithoautotrophically-produced organic matter has the potential to supplement organic matter pools in the subsurface, especially if the surface and subsurface are hydrologically disconnected. Chemolithoautotrophic contributions are less understood for most groundwater and cave ecosystems, especially from ecosystems dominated by sessile filter-feeders that cannot easily move to search for food. Our study focuses on uncovering the microbiology and organic matter contributions in Croatian Dinaric Karst caves, specifically in the Neretva and Lika River basins, that contain the only subterranean serpulid tube worm, *Marifugia cavatica*, the only known cave-adapted freshwater bivalves, *Congeria kusceri* and *Congeria jalzici*, and stygobitic and stygophilic sponges, *Eunapius subterraneus* and *Ephydatia fluviatilis*, respectively.

### Methods

We collected surface water, invertebrates, and representative examples of surface organic matter, as well as subsurface water, stygobionts, biofilms, and sediments from Pukotina u Tunelu Polje Jezero in the Neretva River basin and Markov Ponor and Susik Ponor in the Lika River basin. To evaluate microbial communities, 16S rRNA genes were sequenced, analyzed using mothur to obtain operational taxonomic units (OTUs) at 99% sequence similarity, and classified with the SILVA v138.1 reference database. We used the program FAPROTAX and recently published literature to identify putative

metabolisms for OTUs, focusing on identifying chemolithoautotrophic functions. We measured stable carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope compositions to assess potential food sources for the stygobionts from surface and subsurface materials.

We compared microbial community diversity among caves and sample types using non-metric multidimensional scaling (NMDS) on a Bray-Curtis dissimilarity matrix of rarefied presence/absence data. Analysis of similarity (ANOSIM) on the dissimilarity matrix was used to compare sample type and cave. Welch's t-test was used to compare differences in isotopic composition between surface and caves, and Kruskal-Wallis was used to compare differences among caves. Markov Chain Monte Carlo simulations were employed using mixSIAR v3.1.12, with a chain length of 100000, to calculate the contribution of food sources using a diet tissue discrimination factor of  $\delta^{13}\text{C}=1.2\pm 0.39\%$  and  $\delta^{15}\text{N}=4\pm 0.18\%$ . All analyses were performed in R using vegan (v. 2.6.4) and stats (v. 4.2) packages.

## Results and Discussion

Microbial community composition varied significantly among sample types in each cave (ANOSIM;  $R=0.74$ ,  $p<0.005$ ) but weakly among caves and the surface (ANOSIM;  $R=0.19$ ,  $p=0.019$ , Fig. 1a). Putative chemolithoautotrophs included methylotrophs, dissimilatory nitrate-reducers, sulfur-compound-oxidizers, and hydrogen-oxidizers (Fig. 1 b). Nitrospirales comprised 2.4% to 10.7% of the biofilms but  $\leq 0.2\%$  in water. In Markov Ponor and Susik Ponor, putative methylotrophs (order Methylococcales) comprised 14.0% to 28.3% of the sequence reads in water and 1.2% to 38.7% of reads from biofilms and sediment (Fig. 1b).

We hypothesized that isotopic compositions of filter-feeding stygobiont tissues would point to diets that relied on chemolithoautotrophic carbon.  $\delta^{13}\text{C}$  values for dissolved organic carbon in the caves were statistically similar to those of surface water, at  $-23.9\pm 1.7\%$  ( $n=9$ ), as was dissolved inorganic carbon, at  $-6.4\pm 2.9\%$  ( $n=8$ ).  $\delta^{15}\text{N}$  values from the cave water ranged from 4.7‰ to 13.9‰, whereas  $\delta^{15}\text{N}$  values from the surface was 9.6‰. Cave biofilm  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values ranged, respectively, from -29.8‰ to -27.2‰ (average -28.6‰,  $n=22$ ) and 3.0‰ to 8.2‰ (average 5.8‰,  $n=20$ ). In contrast, photosynthetic organic matter from the surface ranged from  $\delta^{13}\text{C}$  of -45.7‰ to -17.0‰ and  $\delta^{15}\text{N}$  from 2.4‰ to 6.9‰. Surface invertebrates ranged from  $\delta^{13}\text{C}$  of -32.7‰ to -19.0‰ and  $\delta^{15}\text{N}$  of 1.1‰ to 7.5‰. *M. cavatica* tissues from the two river basins did not vary significantly, ranging from  $\delta^{13}\text{C}$  of -32‰ to -33‰, but  $\delta^{15}\text{N}$  varied significantly (Welch's t-test  $p<0.005$ ). The isotopic compositions of *Conger* spp. and the sponges varied significantly between the two river basins.

Different chemolithoautotrophic pathways have the potential to discriminate against  $^{13}\text{C}$  by up to 35‰ or more and  $^{15}\text{N}$  from 0‰ to 18‰. Contributions from such fractionation values were evident in biofilm and stygobiont  $\delta^{13}\text{C}$  values compared to surface organic matter  $\delta^{13}\text{C}$  values. Preliminary mixing model results suggest that allochthonous organic matter contributed to most of the stygobionts' diet, likely due to high flow and input rates during the rainy season prior to collection. However, chemolithoautotrophically-produced

organic matter could contribute up to 10% of some stygobiont diets, depending on the stygobiont and cave system (Fig. 1c). As such, there is potential that in situ chemolithoautotrophically-produced organic matter could serve as a small dietary buffer for sessile stygobionts during changes in surface conditions that affect the water supply and nutrient input. This research has implications for understanding the microbial ecology and diversity of Croatian karst that support endemic fauna and should motivate efforts to protect the watersheds associated with their habitats.

## **Keywords**

stable isotopes, Croatia, Dinaric Karst, resource supply, trophic interactions, microbial, groundwater

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## **Conflicts of interest**

The authors have declared that no competing interests exist.

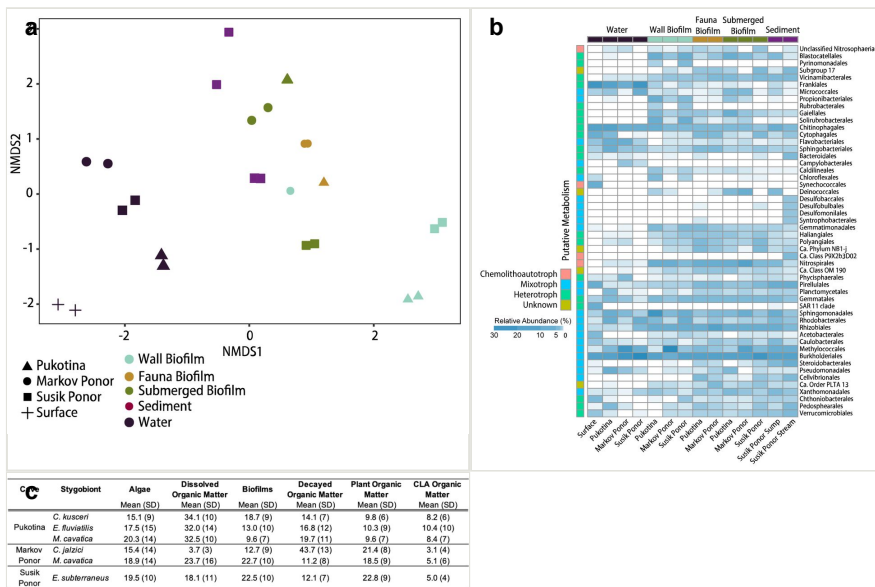


Figure 1.

(A) NMDS plot (stress=0.12) constructed using a Bray-Curtis dissimilarity matrix of microbial communities colored by sample type. Shapes represent different caves. (B) A heatmap of relative abundance of microbial orders with > 1% abundance in at least two samples. Colored bars along rows indicate putative metabolic function. (C) Calculated mixing model contributions for various food sources for stygobionts in percentages, including chemolithoautotrophy.